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THE APPLICATION OF THE PRECIPITIN TECHNIQUE TO THEORIES CONCERNING THE ORIGIN OF VERTEBRATES

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Introduction

Various types of evidence have been employed to determine the relationships between chordates and the different invertebrate groups, and numerous theories have been advanced to derive vertebrates from echinoderms, annelids, and arthropods. These theories have been based on subjective considerations and have resulted from individual interpretations of the value of particular structures in determining descent. At the present time, most workers are agreed that theories which derive chordates from echinoderms are probably correct. However, as recently as 1922, Delsman supported the annelid hypothesis of vertebrate ancestry. The consensus of present-day opinion regarding vertebrate origin is represented in Allee's diphyletic tree which shows probable relationships of the principal groups of the animal kingdom. The principal evidence for this scheme is embryological: (1) the fate of the blastopore which in both echinoderms and chordates does not become the mouth but forms the anus or a nearby structure (neurenteric canal), placing these animals in the group known as Deuterostomia, and which in annelids, arthropods and molluscs becomes the mouth, placing these animals in the group known as Proterostomia; (2) the method of mesoderm and coelom formation, which in deuterostomians is by paired evaginations from the endoderm, forming enterocoelous pouches, and which in proterostomians is by proliferation from two mesoblastic or pole cells, the mesodermal bands later splitting and cavitating to form schizocoelous spaces; and (3) the type of cleavage, which in echinoderms and chordates is, generally speaking, indeterminant, and in annelids, arthropods and molluscs is determinant.

Biochemical confirmation of ideas which derive chordates from echinoderms has come from an analysis of their phosphagens, reported by Needham et al (1932). These substances, so important as sources

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of immediate energy during muscular contraction, are present as creatine phosphoric acid in vertebrates and as arginine phosphoric acid in invertebrates. However, in the sea urchin, *Strongylocentrotus lividus,* and the prochordate, *Balanoglossus salmoneus,* these two phosphagens occur together indicating that the invertebrate showing closest chemical affinity to the prochordate is the echinoderm.

In view of the existing controversy, an objective test which can reveal and express quantitatively the relationships between organisms is desirable in the solution of this problem. In interrelationship studies, morphological and developmental data have been supplemented in recent years by the results obtained from serological reactions. As indicated in a number of researches, the precipitin test is best suited for determining interrelationships of organisms, since it "lends itself admirably to qualitative, quantitative, and *objective* interpretations, which are necessary and fundamental for the evaluation of serological relationships" (Wilhelmi, 1940).

Though never before intensively studied, it seemed that analysis of precipitin reactions between materials of various invertebrates and prochordates would indicate which invertebrate group has the closest physiological and chemical affinity, and presumably, therefore, the closest genetic relationship, to the chordates. This information provides an objective basis for establishing the origin of vertebrates. Accordingly, the purposes of the present investigation are to determine: (1) species-specific titer, thus giving a reference value for the heterologous titers which might be obtained, (2) interrelationships of the several species of invertebrates and prochordates, and (3) relationships between the species of invertebrates and between the species of prochordates.

Materials and Methods

Since three phyla of invertebrates have been involved in theories of vertebrate origin, several species from each phylum (except Arthropoda) were selected, in addition to several species of prochordates, for the preparation of antigens. The following species were employed: Echinodermata—*Asterias forbesi, Arbacia punctulata, Echinarachnius parma, Leptosynapta inhaerens, Thyone briareus;* Annelida—*Arabella opalina, Chaetopterus pergamentaceus, Cirratulus grandis, Glycera dibranchiata, Phascolosoma gouldi;* Arthropoda—*Limulus polyphemus;* Chordata—*Dolichoglossus kowalevskyi, Amaroucium constellatum, Molgula manhattensis, Styela partita.* It will be noted that the habitat of all these animals is marine; hence, species differences are expressions of genetic differences, and those resulting from environmental influences are reduced to a minimum.

Preparation of Antigen

When the whole serum of an animal is employed, the preparation of antigen becomes a simple matter of removing the blood, allowing it to clot, and filtering the serum which separates. However, when the sources are invertebrates, and especially the lower phyla which have no blood, all antigens are necessarily extracts of tissues, and, hence, numerous difficulties are encountered and many precautions must be exercised.

To remove certain contaminating organisms, foreign proteins and debris, the animals when obtained were kept in large crystallizing dishes supplied for a day or so with filtered fresh sea water. Following the preliminary period of exposure to filtered sea water, they were carefully washed through several changes of sterile sea water contained in covered crystallizing dishes, and finally quickly through sterile distilled water to effect a removal of excess salts. This careful washing procedure reduced the bacterial contamination to a minimum, and the long period of washing allowed elimination of fecal material from the alimentary tract, although the alimentary tract was removed in most cases, especially in the annelids. Manipulations in and through the sterile media were done carefully to avoid contamination, sterile forceps being used to effect transfers from one dish of sterile medium to another.

Having reduced contamination, the soft tissues of the living animals were minced on a glass plate or in a shallow glass dish (Petri) and frozen rapidly, using dry ice. Material, once frozen, was kept at this low temperature in a vacuum desiccator (evacuated by a Cenco-Hyvac pump) over anhydrous $CaCl_2$ or $Mg(ClO_4)_2$ until thoroughly dried. As noted in an earlier paper (Wilhelmi, 1940), this freezing-drying, or so-called lyophile, process has certain obvious advantages. Rapid freezing preserves the original structure of labile substances, and the high vacuum causes rapid dehydration and—more important—autorefrigeration to the extent of keeping the materials frozen. Furthermore, the process removes oxygen which could act on antigenic substances to alter their native structure. Desiccation in the frozen state results in dried material which has a spongy texture, and such material is readily pulverized.

After twenty-four hours in the vacuum, the material was pulverized and then returned to the desiccator to insure complete desiccation, indicated when a constant weight was attained. Certain of the annelid materials required a longer time for complete desiccation.

The lipids were removed from the material by repeated extractions with Bloor's mixture, consisting of three parts absolute ether to one part absolute alcohol.

After weighing the lipid-free residue, it was extracted for twelve to twenty-four hours with buffered saline solution maintaining a pH of 7.3, extraction being effected in Erlenmeyer flasks at room temperatures and under constant agitation provided by a mechanical shaker.

After aqueous extraction the material was filtered through sterilized fritted Jena-glass filters, and the clear, sterile filtrate was transferred to sterile, cotton-plugged, small Erlenmeyer flasks and kept in the refrigerator until needed. The residue was thoroughly desiccated and weighed, and the exact amount of material in solution was determined by calculating the difference in weight of residues before and after the aqueous extraction.

Antibody Production

For injection purposes, rabbits of equivalent weights were used after an observational period of several weeks prior to experimentation. Four intravenous injections, one every third day, with gradually increasing dosage, had been found earlier (Wilhelmi, 1940) to be the most satisfactory procedure in the rapid production of high-titered, consistent antisera. This short method of antibody production was followed in the present experiments, but the incubation period following the last injection was increased to twelve or fifteen days.

In the series of four injections, a total of 500 mg. of dry-weight, lipid-free antigen was injected. As in the experiments noted above, this total was divided into doses of increasing amounts, i.e., 25, 75, 150 and 250 mg., respectively, in these series.

After the period of incubation, the blood, withdrawn aseptically by intracardial puncture, using a large, sterile hypodermic syringe fitted with a 19-gauge needle, was placed in sterile, cotton-plugged centrifuge tubes or Erlenmeyer flasks and allowed to clot. After standing in the refrigerator for several hours, the serum was drawn or poured off, usually subsequent to centrifugation. In cases where more than one rabbit was employed in the production of antiserum to a single antigen, the resultant antisera were pooled; the species involved were *Arbacia punctulata, Chaetopterus pergamentaceus,* and *Limulus polyphemus.* Antisera were used immediately for tests or placed in sterile bottles for storage in the refrigerator until needed for the precipitin reaction.

Precipitin Test

In a precipitin tube (8.0 mm., inside diameter), 0.5 cc. of undiluted antiserum was carefully overlaid by 0.5 cc. of appropriately and variously diluted antigen so that a definite interfacial boundary between the

two reagents was maintained. The antigen was brought to the proper concentration by adding sterile saline solution buffered to a pH of 7.3. A standard dilution of each antigen was prepared so that 1.0 gram of dry-weight, lipid-free material would be contained in 100 cc. of saline solution, i.e., 0.01 g./cc. or a dilution of 1 : 100. In homologous tests this standard was diluted to 1 : 1000 for the initial tube, and the dilution in each succeeding tube of a series was doubled, so that a series of antigen dilutions from 1 : 1000, through 1 : 2000, 1 : 4000, etc. up to 1 : 4,096,000 was employed. In the heterologous tests, dilutions from 1 : 100, or in some cases, 1 : 50, were used in the initial tube, and the concentration of antigen was halved in each successive tube. In a positive reaction, a ring or layer of white precipitate occurred at the interphase of antiserum and antigen.

The titer of a reaction is the highest antigen dilution which yields a visible precipitate within one hour at 37° C. when tested with an antiserum, either homologous or heterologous, the amount of antigen being determined on the basis of dry weight of antigenic material actually in solution.

Appropriate control tests were always conducted and involved the use of (1) normal sera and antigen solutions, (2) immune sera and the extracting salt solution, and (3) normal sera and the extractant.

Observations and Results

Table I presents the titers of both homologous and heterologous reactions between the several species of invertebrates and prochordates employed in these experiments. Each titer represents the average of two, or, in many cases, three determinations at 37° C. Negative reactions are indicated by minus signs (—), and tests which were not performed, by blank spaces.

Although rather high, titers of homologous tests cover a wide range, from 1 : 64,000 to 1 : 2,048,000. However, for the most part, the species-specific titer is 1 : 1,024,000 plus or minus one dilution tube. Marked deviations from this value are the titers for *Thyone briareus* among the Echinodermata, and *Arabella opalina* and *Cirratulus grandis* among the Annelida, and, of these, the deviations can be explained in all except the *T. briareus* materials. *Amaroucium constellatum* materials were tested, but a relatively low homologous titer (1 : 32,000) was obtained and the results are not included here since the low titer is explained by crystallization which occurred in the flask containing the antigen, after the injection series had been started; the tautomeric crystallization apparently removed enough antigenic material from solution

TABLE I

Homologous and Heterologous Precipitin Reactions

Number in Parenthesis below Each Titer Indicates Relationship Expressed as Per Cent Value of Homologous Titer

Antigen \ Antiserum	*Dolichoglossus kowalevskyi*	*Styela partita*	*Molgula manhattensis*	*Asterias forbesi*	*Arbacia punctulata*	*Echinarachnius parma*	*Thyone briareus*	*Leptosynapta inhaerens*	*Limulus polyphemus*	*Arabella opalina*	*Glycera dibranchiata*	*Chaetopterus pergamentaceus*	*Cirratulus grandis*	*Phascolosoma gouldi*
Dolichoglossus kowalevskyi	512,000 (*100*)	16,000 (1.562)	64,000 (3.125)	4,000 (0.195)	1,500 (0.073)		2,000 (0.78)	8,000 (0.78)	— (0.000)			80 (0.0078)		100 (0.0195)
Styela partita	8,000 (1.562)	1,024,000 (*100*)			4,000 (0.195)			1,000 (0.098)	400 (0.0195)			200 (0.0195)		— (0.000)
Molgula manhattensis	32,000 (6.25)		2,048,000 (*100*)	2,000 (0.098)	4,000 (0.195)		1,000 (0.39)	1,000 (0.098)	150 (0.0074)	— (0.000)	— (0.000)	150 (0.0146)		— (0.000)
Asterias forbesi			3,000 (0.146)	2,048,000 (*100*)	64,000 (3.125)									
Arbacia punctulata	2,000 (0.39)	4,000 (0.39)	4,000 (0.195)	32,000 (1.562)	2,048,000 (*100*)	32,000 (6.25)		21,300 (2.08)	100 (0.005)			200 (0.0195)		100 (0.0195)
Echinarachnius parma		1,000 (0.098)			128,000 (6.25)	512,000 (*100*)			100 (0.005)	— (0.000)	— (0.000)	300 (0.0293)		
Thyone briareus	3,000 (0.586)						256,000 (*100*)	64,000 (6.25)						
Leptosynapta inhaerens	8,000 (1.562)	2,000 (0.195)			32,000 (1.562)			1,024,000 (*100*)						
Limulus polyphemus	300 (0.0586)	150 (0.0146)	100 (0.005)		50 (0.0024)			200 (0.0195)	2,048,000 (*100*)	— (0.000)		4,000 (0.39)	800 (0.3125)	
Arabella opalina						— (0.000)			4,000 (0.195)	64,000 (*100*)				
Glycera dibranchiata											512,000 (*100*)	16,000 (1.562)		
Chaetopterus pergamentaceus	200 (0.039)	200 (0.0195)	400 (0.0195)		200 (0.0098)	100 (0.0195)			4,000 (0.195)		12,000 (2.344)	1,024,000 (*100*)		
Cirratulus grandis									2,000 (0.098)		16,000 (3.125)	8,000 (0.78)	256,000 (*100*)	
Phascolosoma gouldi	75 (0.015)	75 (0.0073)			— (0.000)									512,000 (*100*)

so that not only was considerably less than 500 mg. injected but also the dilution in the last tube yielding a positive reaction was probably greater than 1 : 32,000.

Wherever available materials permitted, reciprocal tests were conducted, and, in general, such tests confirmed the original ones, i.e., were of the same order of magnitude. Such confirmation is imperative if the data and resultant interpretations are to be significant. As Boyden (1926) so very appropriately stated, "it would seem then that this principle of reciprocal relationships could be used as a test of the truth of the values obtained in the precipitin reaction. . . . Only those values which check within the limits of error of the reaction may then be taken."

Titers of cross-reactions between members of the various invertebrate phyla and the Prochordata were, in general, much higher in the case of echinoderm-prochordate reactions than in annelid-prochordate and/or arthropod-prochordate tests. The titers of heterologous reactions between annelid and arthropod materials were of the same order of magnitude as those between echinoderm and prochordate materials.

In Table I, the figures in parentheses represent the titration data converted to percentages. As in reports by other authors, the homologous titer of a particular antiserum is taken as 100 per cent and the titers of all heterologous tests with that antiserum are expressed as percentages of the homologous titers. For example, *Leptosynapta inhaerens* antigen when tested against *Dolichoglossus kowalevskyi* antiserum yields a titer of 1 : 8000; the homologous titer of *D. kowalevskyi* antiserum being 1 : 512,000, the percentage value becomes $\frac{8000}{512{,}000}$ or 1.562 per cent.

Discussion

Homologous serological reactions of high titer would be necessary if heterologous reactions between such distantly related groups as prochordates and any of the invertebrate phyla are to be obtained. Hence, large amounts of antigen were injected in the hope that a maximum antibody response of the rabbit would be elicited, and, from the results, it is apparent that a strong, though perhaps not maximum, antibody response was evoked. With three exceptions, the titer of homologous reactions was 1 : 1,024,000 plus or minus one dilution tube. No explanation is apparent for the aberrant titer of 1 : 256,000 obtained with *Thyone briareus* materials. However, in both *Arabella opalina* and *Cirratulus grandis*, the lower titers seem related to the difficulty experienced in desiccating the animals; at least, there is a possibility that antigenic materials

were altered because desiccation required two or three days, as compared with the usual eighteen to twenty-four hours, and the worms did not remain frozen until dehydrated. Of course, individual variations in the ability of rabbits to produce antibodies may be sufficient to cause these variations in titers, but the rabbits used were progeny of a single pair and were of equivalent weights, which would seem to reduce the variability as a result of genetic factors.

In spite of the wide range in homologous titers, the results in the present work are rather uniform, when compared with those reported in certain other investigations, since the exceptions in all but one instance can be satisfactorily explained and the deviation in that one instance is only one dilution tube removed from the generally accepted limit of error. Although the lipids have not been employed as antigens in direct tests, it is probable that extraction of lipids is largely responsible for the uniformity of results. It seems appropriate, therefore, to reiterate and to reemphasize certain observations concerning the specificity of these haptenic components, although a more complete account was presented in an earlier report (Wilhelmi, 1940). While the serological and chemical properties of proteins closely parallel the zoological relationships of organisms from which they are obtained, i.e., the more similar the proteins in organisms, the closer the phylogenetic relationships, the lipids of closely related species, or, indeed, even in different organs of the same species, may differ considerably, and the lipids of distantly related species may be very similar, if not identical, immunochemically. Boyden's (1936) observation that *"the convergent reactions obtained with antisera to native serum . . . disappeared* following ether extraction" and results from the present experiments demonstrate that the group-specific or non-specific character of lipids demands their complete removal from antigenic materials designed to determine zoological or phylogenetic relationships of organisms.

Serological methods have been increasingly stressed in experimental work designed to clarify phylogenetic relationships of animals, at least within more or less restricted groups, such as orders, classes, and phyla. Among invertebrate Metazoa, research involving serological reactions and relationships has been rather limited, being confined for the most part to experiments with helminths. Erhardt (1931) reviewed the serological studies which have involved interrelationships of animals, but in very few cases were positive interphylar reactions noted. In Nuttall's (1904) classic treatise, three doubtfully positive reactions between anti-xiphosuran serum and fish serum were reported, as well as a few positive crustacean-amphibian and crustacean-reptilian reactions. Boyden (1934,

1936) has recorded a serological study of the relationships of some invertebrates, but, unfortunately, results in the form of titers, etc. were not noted, although antigens of Nemertinea, Annelida, Mollusca, Arthropoda, Echinodermata, and Chordata had been collected and injected. Boyden (1934) stated, "These tests clearly indicate that significant data regarding the interrelationships of the members of the higher invertebrate phyla may be obtained through the use of the precipitin reaction. They indicate furthermore that interphylar reactions may even be obtained—a point of some phylogenetic importance."

In spite of general agreement, based on morphological and developmental data, that echinoderms and chordates arose from a common ancestral stock, the origin of chordates, and especially of vertebrates, from the invertebrates has been the subject of considerable speculation and theorization. Many zoologists still contend that theories which derive the vertebrates from the Annelida are most plausible; indeed, as noted above, Delsman (1922) published a modified annelid theory to explain the origin of chordates. The results of the heterologous precipitin tests in the present experiments indicate clearly that echinoderms are the invertebrates with the closest chemical and physiological affinity to prochordates. The titers of echinoderm-prochordate tests are several times greater than the highest titer exhibited by annelid-prochordate or arthropod-prochordate reactions, being, in some cases, twenty times the titer of the proterostomian-prochordate tests, i.e., five dilution tubes removed from the latter. It is noteworthy, however, that the materials of Annelida and Arthropoda do react with those of Prochordata, even if at very low titers.

Results available at present indicate that, of the echinoderms tested, the holothurians are most closely related to the prochordates, and, of the latter group, to the hemichordates, since the *Leptosynapta inhaerens-Dolichoglossus kowalevskyi* reactions yield the highest interphylar titers, both the original tests and the confirmatory reciprocals. However, more tests are necessary before a final appraisal or judgment can be given.

If the Proterostomia separated from the Deuterostomia early in the evolutionary history of animals, then one should expect cross-reactions to occur between annelid and arthropod materials at higher antigen dilutions than between either of these and the deuterostomian materials. Such higher titers were obtained, thus speaking for the closer phylogenetic relationship between Annelida and Arthropoda than between either phylum and the Deuterostomia.

Intraphylar heterologous tests were made for purposes of comparison with the interphylar reactions. The available results demonstrate much

higher titers in intraphylar than in interphylar reactions. However, relationships within restricted groups were not the primary interest in these experiments, and, furthermore, lack of materials has prevented an extension of the researches to include more intraphylar tests. These preliminary results will have to be supplemented before the results of precipitin tests can be applied to phylogenesis within the restricted groups.

When the experimental results are converted to percentages, many of the differences are reduced and many of the discrepancies disappear completely. For example, the titer of the reaction between *Arbacia punctulata* antigen and *Echinarachnius parma* antiserum was 1:32,000, while in the reciprocal tests the titer was 1:128,000; yet, the resultant percentage values are 6.25 in both instances. Also, as may have been noted, the titers of reactions involving *Styela partita* antigen and *Leptosynapta inhaerens* antiserum (1:1000) and *S. partita* antigen and *Limulus polyphemus* antiserum (1:400) are rather close (a difference of slightly more than one dilution tube), although the percentages are very different, the prochordate-echinoderm value (0.098) being five times greater than the prochordate-arthropod value (0.0195). One of the most striking interpretations derived from percentage data is the enhancement given to the suggestion that holothurians (Echinodermata) and hemichordates (Prochordata) are most closely related serologically; indeed, the percentage values between these groups are at least twice as great as the nearest echinoid-prochordate or asteroid-prochordate value. Treating titration data on a percentage basis is necessary in attempts to compare and to correlate experimental results reported by authors whose techniques and procedures have differed. However, in spite of the rather general use of percentages and in spite of the enhancement given to certain suggestions and interpretations derived from analysis of the titers in the present work, it seems advisable to analyze and interpret the results of experiments directly from titration data, and, in future research, to attempt the elimination of discrepancies between titers of comparable reactions by further refinements in technique.

Summary and Conclusions

The precipitin reaction, demonstrated to be valuable in determining phylogenetic relationships of animals within restricted groups, was applied to distantly related groups among the invertebrates and chordates to test, by this objective method, the various theories concerning the origin of vertebrates. From the results it is apparent that serologically, as well as morphologically and developmentally, the chordates are more closely related to the echinoderms than to any other group of inverte-

brates tested, indicating that chordates evolved from animals which also gave rise to present-day echinoderms.

Consistent results, confirmed by reciprocal tests, were obtained by injection of aqueous solutions containing five hundred milligrams of dried antigenic material from which the lipids had been removed. The rather uniform results may be attributed to the following important steps: vacuum desiccation of materials in the frozen state (lyophilization), preliminary removal of lipids from desiccated material to exclude the possibility of group reactions, control of hydrogen ion concentration during extractions and tests, control of temperatures at which reactions are performed, and knowledge of the actual amount of material in the antigenic solutions.

The titer of a reaction is the highest antigen dilution which gives a visible precipitate when tested with an antiserum, the amount of antigen being determined on the basis of dry weight of antigenic material actually in solution. With three exceptions, the titer of homologous reactions is 1 : 1,024,000 plus or minus one dilution tube, and heterologous titers never exceeded homologous ones.

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HISTOLOGY OF THE RADULA PROTRACTOR MUSCLES OF BUSYCON CANALICULATUM LINN.

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The muscle fibers of the molluscs have perhaps been more completely studied than those of any other invertebrate group, yet there is no real agreement on their structure. One explanation for this confusion is the apparent presence in most molluscs of several types of muscle fibers. Roskin (1935) describes six different types of smooth muscle fibers and a primitive type of cross-striated muscle in five closely related species of pteropod molluscs. Marceau (1937) in a study of the adductor muscles of more than twenty bivalve molluscs describes five rather distinct types as well as a number of transition or combination forms of the five types.

Perhaps the most serious confusion in the literature on molluscan muscle has resulted from the dispute as to the presence or absence of cross-striated, obliquely striated, and spirally striated muscle fibers. Schwalbe (1869) believed that the arrangement of granules might give the appearance of striations. Schwalbe, however, did describe a type of double oblique striation in which the contractile substance appeared as a diamond-shaped design. Paneth (1885) stated that the muscles of the heteropods and pteropods were in all essentials like the muscles of the vertebrates. Fol (1888) drew the conclusion that the appearance of cross-striation or the double oblique striation of Schwalbe might result from the spiral twisting of the myofibrils. He believed that the only type of muscle present in the molluscs was smooth.

Merton (1911) found both smooth and striated muscle present in the molluscs, but he considered the striated muscle to be different from that of higher animals since the striations disappeared in the resting state. He also gave several explanations for the manner in which smooth muscle fibers might under certain conditions resemble cross-striated muscle fibers. In the first place, the contracted fiber could be fixed so that the myofibrils occurred as wavy lines. The curve of the fibril would stain heavily, the straight portion lightly and thus give the appearance of cross-striations. In the second place, under certain conditions of fixation and staining, the connective tissue fibers running at right angles to the muscle fibers might be confused with cross-striations. Finally, he uses the ear-

lier explanation of Schwalbe that the granules might be aligned to simulate cross-striations.

Plenk (1922, 1924) states that all of the fibers in the masticating apparatus of the snail are cross-striated although he does not deny the presence of smooth muscle in other parts of the body. He believes that apparently smooth muscle fibers are really cross-striated fibers in a certain functional state and that a much more widespread existence of striated muscle in the animal must be assumed. The oblique and spiral striations described by earlier workers are explained by him as distortions resulting from the rigors of fixation and preparation.

The trend of later investigations (Roskin 1925, 1935, Marceau 1937), seems to be the recognition of the presence of different types of smooth and striated fibers.

For the sake of clarity I have limited the present investigation to a detailed study of the radula protractor muscles of *Busycon*.

Materials and Methods

The radula protractor muscles of *Busycon canaliculatum* consist of three pairs of flattened bands on the ventral side of the buccal mass. The median pair are somewhat thicker than those which cover the horns of the odontophoral cartilage. In the resting state the muscles are about 2.5 cm. long. The median pair are about 1.5 mm. wide and 1 mm. thick; the two smaller pair are about 1 mm. wide and .75 mm. thick.

The muscles were fixed in varying states of contraction in Bouin's and Helly's fixing fluids. They were dehydrated in Cellosolve, cleared in xylene, imbedded in paraffin, and sectioned at 5 and 10 micra. The sections were stained with Heidenhain's iron haematoxylin, Mallory's phosphotungstic acid haematoxylin, and Dominici's staining mixture.

The muscle fibers were dissociated with 35 per cent KOH.

Results

The muscles consist essentially of densely packed, spindle-shaped, uninucleate, unstriated fibers which have an average length of about 1 mm. Occasional fibers are found which attain a length of about 1.7 mm. and others which may be as short as 360 micra. The average diameter in contraction is about 6 micra and in extension about 3.5 micra.

In cross-section the fibers appear cylindrical, hemicylindrical, or angular (Figs. 1 and 2). The fibers possess two clearly defined zones; a rather heavily staining cortex and a lightly staining medulla. A number of comparatively coarse myofibrils are scattered throughout the cortical

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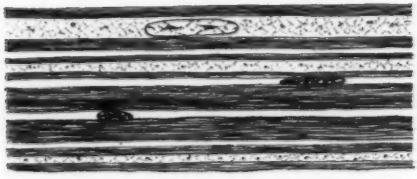

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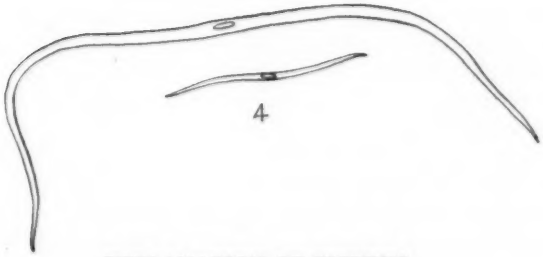

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EXPLANATION OF FIGURES

All drawings were made with the aid of a camera lucida.

FIG. 1. Cross-section of a small portion of an extended muscle stained with Mallory's phosphotungstic acid haematoxylin showing the shape and arrangement of fibers, the fibrillar cortex, and coarsely granular-vesicular axillary region of each fiber. × 1750.

FIG. 2. Cross-section of a small portion of a contracted muscle stained with Heidenhain's iron haematoxylin. The coarse myofibrils are not visible with this stain but areas of contraction stain heavily. × 1750.

FIG. 3. Longitudinal section of a portion of an extended muscle stained with Mallory's phosphotungstic acid haematoxylin showing the fibrillar cortex, the coarsely granular-vesicular medullary portion. The nucleus of a muscle fiber is shown lying in the medullary region and connective tissue nuclei are shown lying between the muscle fibers. × 875.

FIG. 4. A large and a small fiber from a preparation of macerated muscle. × 88.

zone with a slight concentration at the boundary of the cortex and medulla (Figs. 1 and 3). The myofibrillae are neither as fine nor as abundant as those observed in the muscles of *Phascolosoma* (Olson, 1940). The fibrils are visible only with Mallory's phosphotungstic acid haematoxylin, in which respect they differ from those of *Phascolosoma*. With Heidenhain's iron haematoxylin it was possible to see the darkly staining regions of contraction in the cortical zone of the fibers. It is rather peculiar that these are not clearly defined nodes, involving a whole segment of a fiber as is true in *Phascolosoma*, but may appear singly or as scattered patches in cross-sectional views of the fiber (Fig. 2). In longitudinal sections they appear as elongated tapering areas.

The medulla contains large lightly staining granules held in a vesicular network. There are no fibrils present. The elongated nucleus occupies a more or less central position in the medulla. Frequently the fiber shows a definite enlargement at the region of the nucleus.

Similar fibers have been described for other molluscs. Schwalbe (1869) described a number of gastropod muscles which showed cortical and medullary zones. List (1887) described such muscle fibers in *Tethyls;* Agersborg (1923) in *Melibe;* Roskin (1935) in five species of pteropod molluscs; and Marceau (1937) in a number of the species which he studied.

Wackwitz (1892), describing the muscle fibers of heteropods and pteropods, states that the material in the medullary zone appears sometimes granular, sometimes as a vacuolated network, and that the quantity appears to be related to the activity of the fiber.

Schumann (1911) saw in the retractor muscle of *Gadinia* a lightly staining central core and a fibrillated cortex. He also described a type of cross-striation in occasional fibers. Incidental observations which I made on the radula retractor muscles of *Busycon* indicate that they have a structure identical with that of the radula protractor muscles.

Fibers possessing a fibrillated cortical zone and a clear medulla have been observed in other invertebrates besides the molluscs (Andreae 1881, in *Sipunculus;* Apel 1885 in *Priapulus* and *Holicryptus;* and Jameson 1889 in *Thalassema*).

As in the sheets of vertebrate smooth muscle, connective tissue occurs very sparsely between the individual fibers. Unlike vertebrate smooth muscle, however, connective tissue nuclei are found in the interstices between muscle cells (Fig. 3). The proportion of connective tissue and tissue space is somewhat less than in the retractor muscles of *Phascolosoma* (Olson, 1940) and considerably less than in the retractor muscles of *Thyone* (Olson, 1938).

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SEASONAL GONADAL CHANGES IN THE ADULT OYSTERS, OSTREA VIRGINICA, OF LONG ISLAND SOUND

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INTRODUCTION

Gonads of the American oyster, *Ostrea virginica* Gmelin, undergo pronounced changes during the year. Such seasonal changes have been noted in several other Pelecypod mollusks, gonads of which have been studied throughout the annual cycle (Coe, 1932*a* and 1936*a*; Orton, 1933; Roughley, 1933; Loosanoff, 1937*a*; Coe and Turner, 1938). In the ripe adult oyster the gonad is the largest, most conspicuous organ, forming one continuous mass around the folds of the digestive tract and digestive gland, giving the oyster a typical cream-colored appearance. In some years, when gonads are especially well developed, the thickness of the gonadal layer before spawning may reach 1.0 centimeter. During the spawning period, simultaneously with the discharge of gametes, the thickness of the gonadal layer gradually decreases, and in spent oysters the layer becomes extremely thin. Naturally such radical changes, which usually correspond to definite seasons of the year, must markedly affect the physiological behavior of the oyster as well as the chemical composition of its body. Therefore, better knowledge and understanding of the changes occurring in gonads of oysters during different seasons of the year should be helpful in the interpretation of other seasonal phenomena. With this in view a systematic description of seasonal gonadal changes of the adult oysters is offered in this article.

The bulk of material used in these studies was collected from the oyster beds established by the author in Long Island Sound near Milford, Connecticut. These beds were located at different depths ranging from mean low water to 40 feet. Samples were collected over a period of several years at bi-weekly intervals, except in summer when they were taken at least once a week. In all cases the oysters comprising the samples were from four to six years old. Soon after the collection of samples, the oysters were opened and pieces of their gonad tissue placed in a fixing solution, and later prepared for histological studies.

In discussing the changes occurring in the gonads of oysters during

the different seasons of the year, it will be convenient to begin with the discussion of conditions as observed to exist in the winter.

The writer wishes to express his thanks to Mr. James B. Engle for taking the photomicrographs shown in this article.

Winter or Inactive Stage

During the cold season, usually beginning with December, the oysters remain in a dormant state. Examination of hibernating oysters shows that during that period their gonads are also in the state of quiescence. The gonad follicles are few in number and they are scattered in the form of small islands throughout the vesicular connective tissue in the area between the body wall and the digestive gland. Most of the follicles are found in that portion of the connective tissue which lies near the genital ducts, the cilia of which remain easily distinguishable during the winter stage (Fig. 1). The follicles are connected with others by fine strands of connective tissue.

The vesicular connective cells are present in large numbers in the area confined between the genital ducts and the digestive gland. These cells are often seen completely surrounding the gonadal follicles and filling the space between them (Fig. 2). In some cases, groups of phagocytes may be noted infiltrating between the vesicular connective tissue cells, approaching the follicles. Such amoeboid cells with darkly stained nuclei are also frequently observed inside the lumina of the follicles where they may be seen grouped around pycnotic cells, apparently performing phagocytic functions.

The follicles of the winter gonad are small in size and almost devoid of lumina. The sex cells usually form but a single row along the follicular wall, the inner layer of which consists of germinal epithelium. Even during the winter stage the sexes are distinguished from each other because of the large number of young ovocytes present in the female gonad. Such ovocytes are easily detected because of their large clean nucleus and granular cytoplasm. These, as well as the indifferent cells, are always prominent in the wintering gonad of a female oyster (Fig. 1).

PLATE I

Explanation of Figures

Fig. 1. Winter gonad of female oyster showing small follicles containing young ovocytes. Part of genital duct is visible. × 225

Fig. 2. Winter gonad of male oyster. Follicles are separated by cells of vesicular connective tissue. Many phagocytes are present. × 225

Fig. 3. Gonad of female oyster showing the beginning of early spring growth. × 125

Fig. 4. Gonad of male oyster in early spring. × 125

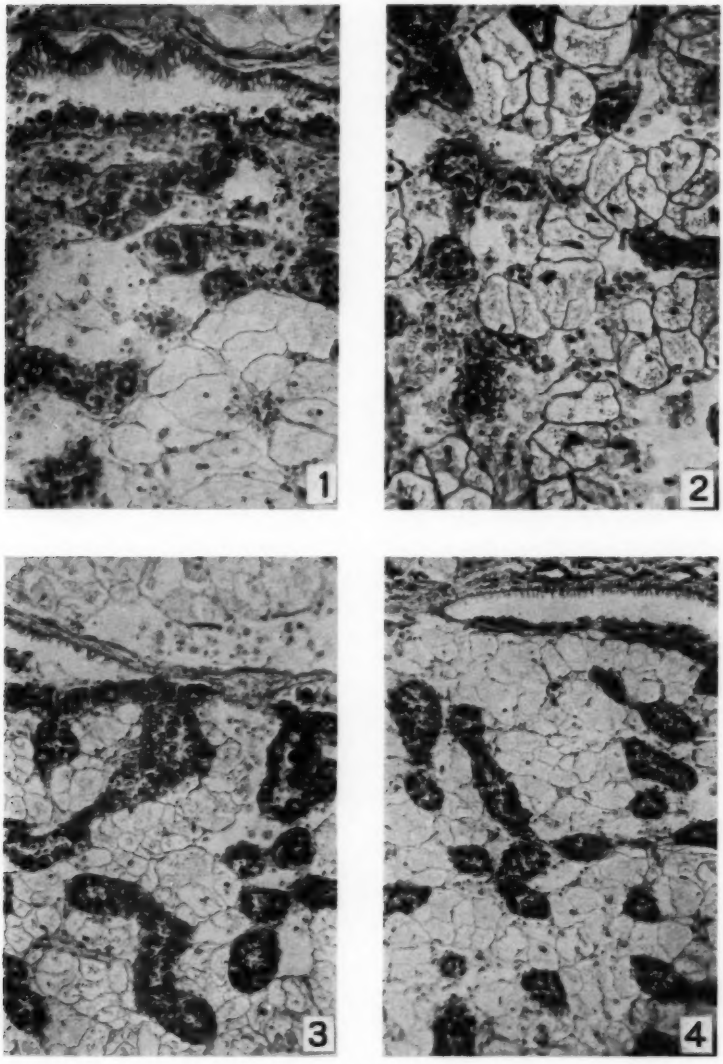

PLATE I

In males (Fig. 2) the follicles are also small and are located throughout the connective tissue in the same way as described for females. They contain indifferent residual cells, spermatogonia and occasionally very few primary spermatocytes. At this, as well as at all other times, when the sex of the oyster can be distinguished, many adult animals of both sexes continue to indicate the unstable character of their gonads. This is especially evident in the males where small ovocytes may be often present along the follicular walls. In the females, groups of cells resembling those of early stages of spermatogenesis may sometimes be detected even in ripe ovaries. Coe (1932*b*) reported similar observations in the case of young oysters, and later (1936*b*) discussed at length the question of the instability of sex in many mollusks.

Spring Development and Prespawning Stage

Early in April, with the arrival of warmer weather, the gonad follicles in some oysters may begin to show an increase in size, and in the number of cells. Such a growth is usually very insignificant. As a rule, a majority of oysters examined at that time showed that their gonads still were in the state resembling the winter condition. Later in April, however, the follicles of many oysters of both sexes slowly begin to enlarge. In females (Fig. 3) young ovocytes begin to grow extending toward the center of the follicles, and in males (Fig. 4) proliferation and anastomosis of the follicles also become more apparent. They now contain comparatively large numbers of primary and secondary spermatocytes, and in some cases, even spermatids are formed.

In May, soon after the water temperature reaches and passes 10.0° C., very rapid growth and ramification of gonadal follicles commence. They are seen anastomosing through the connective tissue from the region of the genital ducts toward the liver and also in the direction of each other (Figs. 5 and 6). In follicles of the female oysters young ovocytes become larger and more numerous. In males spermatogenesis proceeds at quite a rapid pace, and in many animals spermatids and spermatozoa are already formed in large numbers. In some cases apparently ripe sper-

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PLATE II

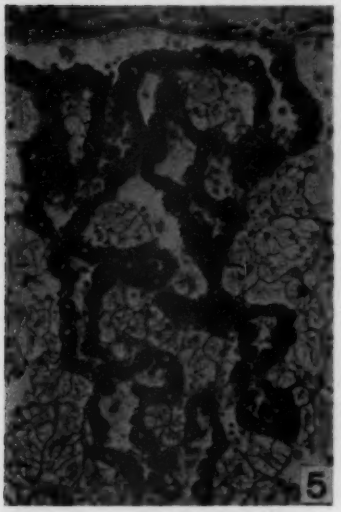
Explanation of Figures

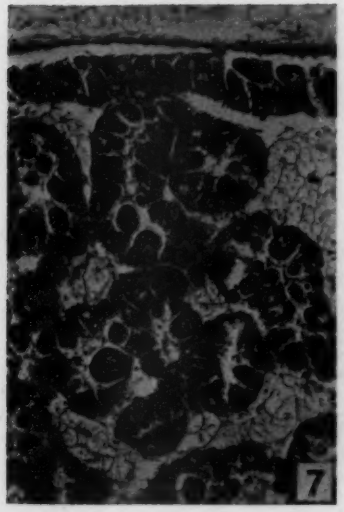
Fig. 5. Gonad of female oyster in May showing rapid growth and ramification of the follicles. × 125

Fig. 6. Male gonad in May. Ripe spermatozoa are already formed. Cells of the connective tissue are gradually disappearing. × 125

Fig. 7. Gonad of female oyster approaching ripeness. × 125

Fig. 8. Gonad of male oyster of nearly ripe stage. Spermatozoa are very numerous. × 125





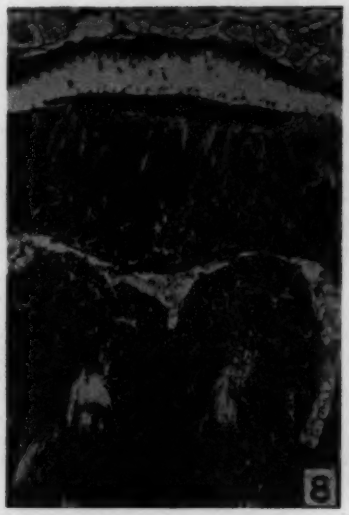


PLATE II

matozoa are noticed entering the genital ducts which are situated beneath the surface epithelium of the oyster body. One of the inner surfaces of the ducts, nearest the body wall, is lined with ciliated epithelium, while the surface facing the gonad is lined with germinal epithelium which gives rise either to male or female sex cells, depending upon the sex of an individual oyster.

In late spring the vesicular connective tissue surrounding the follicles occupies considerably less space than earlier in the season. Roughley (1933) working on the Australian oyster, *O. commercialis,* expressed the opinion that such connective tissue is continually absorbed by the follicles, and is used for the nourishment of the developing sex cells. Coe and Turner (1938) also attribute nutritive functions to such cells. The writer is inclined to accept the same point of view in regard to the connective tissue surrounding the developing gonadal follicles of the adult oysters. As can be seen from figures 3 to 7 inclusive, the connective tissue lies in close contact with the walls of the follicles to which developing sex cells are attached. The nutritive substances contained within the connective cells are probably diffused through the cellular membrane and are assimilated by the sex cells. Such a view merits consideration because in many areas of the gonadal space the connective tissue cells are the only ones surrounding the follicles. The other type of cells, which in some Pelecypoda perform phagocytic-nutritive functions (Loosanoff, 1937*b*), are either absent or found in too small numbers to be considered of importance.

During the first part of June gametogenesis proceeds at a very rapid rate. The gonadal follicles ramify rapidly, and the development of gametes of both sexes is very fast. The vesicular connective tissue, which was so prominent in the spring, begins to disappear (Figs. 7 and 8). In females the ovocytes show pronounced development and some of these cells acquire almost a mature appearance (Fig. 7). In males the sper-

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PLATE III

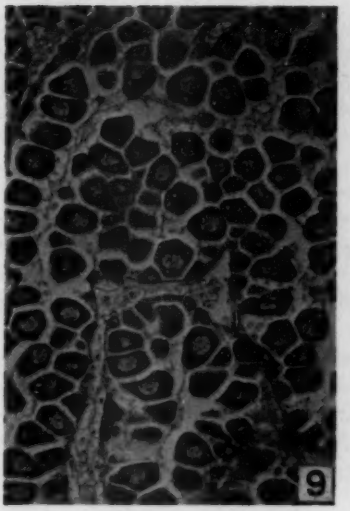
Explanation of Figures

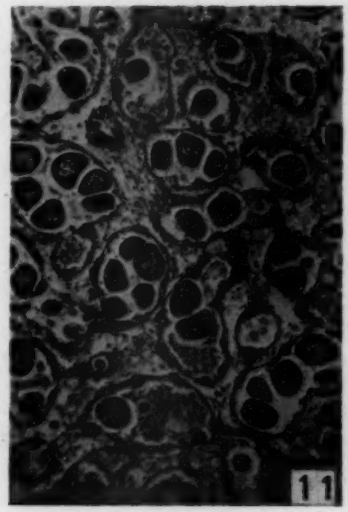
Fig. 9. Section of gonad of ripe female oyster. × 125

Fig. 10. Section of gonad of ripe male oyster. Cells of connective tissue are virtually absent. Mature spermatozoa occupy the follicles. × 125

Fig. 11. Section of gonad of partially spawned female oyster. The follicles are in contracted state. Numerous phagocytic cells are present. Cells of vesicular connective tissue reappearing. × 125

Fig. 12. Gonad of female oyster at the end of spawning. Gonadal layer, confined between the genital duct and digestive gland, is very thin. Phagocytic cells are numerous and active. Gonadal follicles are shrinking. Few eggs are in genital duct on their way to be discharged. Large numbers of connective tissue cells are seen near the section of digestive gland. × 225





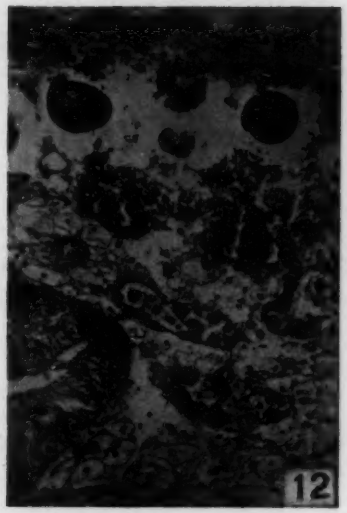


PLATE III

matozoa are more numerous than any other stages of spermatogenesis (Fig. 8).

Toward the end of June, many oysters reach a fully ripe stage. Branches of anastomosing follicles reach the region of the digestive gland, and are separated from it only by a thin layer composed of a few cells of connective tissue. Follicles are now large and distended. In females they contain mainly ripe eggs, although young ovocytes and ovogonia are also present along the follicular walls (Fig. 9). The majority of these cells develop later in the season. As a rule, newly formed follicles in the region near the digestive gland contain more cells in the early stages of gametogenesis than the older follicles located nearer the genital ducts. The quantity of the interfollicular vesicular connective tissue diminishes further. Phagocytic cells can be seen outside the follicles, but usually none are found in the lumina.

In males the central part of the follicles is occupied by large numbers of mature spermatozoa (Fig. 10). Masses of them may be seen protruding into the genital ducts, ready for discharge. The cells at the earlier stages of spermatogenesis, which are usually found near the follicular walls, are few in number.

Partially Spawned Stage

At the proper time, upon receiving certain stimuli among which temperature and chemical impulses are known to be of importance, the oysters begin to discharge their spawn into surrounding water. In Long Island Sound, this usually happens at the end of June or during the first week of July (Loosanoff and Engle, 1940). As a rule, an individual oyster does not discharge its entire content of sex cells at one time. Usually the spawning is extended through many days or even weeks.

The gonads of partially spawned oysters are always easily recognizable. They are characterized by the shrinkage of the follicles from which some of the eggs have already been discharged (Fig. 11). The remaining undischarged eggs are closely grouped together. In males, the central portion of the follicles is often empty because of the recent discharge of large quantities of ripe gametes. Usually almost the entire content of the follicles consists of mature spermatozoa, although a few of the cells of the earlier stages of spermatogenesis can always be found near the follicular walls. In both sexes numerous phagocytic cells appear in interfollicular spaces, and some of them proceed to invade the follicles.

A few cells of the vesicular connective tissue may be seen appearing in the newly formed interfollicular spaces of shrinking gonads.

Advanced Post-Spawning Stage

As the spawning progresses and the oysters enter more advanced stages, a still greater shrinkage of follicles occurs (Fig. 12). There are but few undischarged eggs left, and many follicles are entirely free of them. Phagocytic cells appear in very large numbers, both inside and outside the follicles. They are engaged in devouring and cytolyzing remnants of undischarged gametes and pycnotic cells. At this stage resorption of the gonads proceeds very rapidly and soon, emptied follicles begin to acquire an elongated form. Finally, the lumen almost entirely disappears and the walls of the follicles come virtually in contact with each other. The gonadal layer rapidly diminishes in thickness and the distance between the surface epithelium and the digestive gland of the oyster becomes very small. In the majority of cases examined, cytolysis and the resorption of gonads were completed by the end of October.

It was already mentioned that during the ripe stage there are but few cells of the connective tissue found in the interfollicular spaces. Such cells, however, are found, at that time, in large numbers between the tubular folds of the digestive gland. In the advanced spawning stage, following the invasion of phagocytes, the cells of the connective tissue of the digestive gland region begin to proliferate. Soon masses of these cells may be seen penetrating into the interfollicular spaces gradually surrounding the shrinking follicles. The number of these cells increases parallel with the resorption of gonads, and they reach the greatest abundance at the time when the oysters enter into the winter stage.

Indifferent Stage

As the resorption of the undischarged eggs and spermatozoa is completed, the gonads gradually enter into the indifferent phase. The follicles become very small in size, and are separated from each other by large masses of connective tissue. The follicles are free of all but indifferent sex cells, and as a result it is impossible, at this stage, to determine the sex of an individual. It is thought, therefore, that sex reversal, which is known to occur in American oysters (Burkenroad, 1931; Galtsoff, 1937, and others), takes place during this stage, when sexuality of an oyster is least defined.

Sex Differentiation Stage

After the end of the indifferent period, which usually takes place in late October, slight gametogenic activities are occurring in the oysters. In females numerous ovogonia and small ovocytes are developing along

the follicular wall, while in males a few primary spermatocytes may be found in some follicles. In both sexes the follicles show slight expansion, and animals of different sexes are again easily distinguishable. The fall gametogenesis is, however, of short duration, and as compared with that of the spring, is much less active.

With the arrival of cold weather, the oysters enter into the hibernation period, and their gonads remain in a dormant stage until the next spring.

Discussion

Studies of the changes taking place in the gonads of adult oysters indicate that during the annual cycle these animals experience two periods of gametogenetic activities. The first of these occurs in the autumn, immediately after the indifferent period. It is, however, of very short duration, being interrupted by the onset of low temperature which induces hibernation of the oysters. During the autumn period the cells of only the early stages of gametogenesis are formed. The second period occurs in the spring and early summer and during it the gametogenesis is completed. These observations are in agreement with those of Coe (1932*b*) who reported that in young individuals of *O. virginica* of the Long Island Sound and Woods Hole region, the process of spermatogenesis does not continue through the winter, and no spermatozoa are formed before spring.

The observations made during these studies point to the conclusion that, as first suggested by the author (Loosanoff, 1936) sex reversal, when it occurs, takes place during the indifferent stage when the sexuality of an individual is the least apparent. During this stage, which is of very brief duration, the oyster gonad contains undifferentiated cells only. The further development of these cells, along male or female lines, is probably influenced during the indifferent period by some factors, the nature of which is not very well understood at present. Later in the season, regardless of the fact that gonads still retain their bisexual potencies, the sexes are already strongly defined, and, therefore, it is very improbable that reversal of sex can occur then.

In comparing seasonal gonadal changes of *O. virginica* with those of other species of the oviparous type of the genus *Ostrea,* it will be noted that, in general, they are rather similar. Amemiya (1929) found that in the Japanese oyster, *O. gigas,* the chief gametogenetic activities occur mainly in spring. A similar observation is reported on *O. commercialis* (Roughley, 1933), in which case the gonads are in a resting stage in winter time.

If *O. virginica* is compared with some pelecypods, other than oysters, significant differences may be noted in certain stages of the annual cycle. For example, in the hard shell clam, *V. mercenaria,* contrary to the conditions observed in the oyster, the most active period of gametogenesis and the production of gametes occur in the autumn and early winter. Morphologically ripe spermatozoa and ova are retained by the clam through the winter (Loosanoff, 1937*a*, 1937*b*).

Observations presented in this article describe the gonadal changes that occur at certain seasons of the year in the majority of oyster population of New England waters. Often, however, individuals found in the samples may differ greatly in their conditions from the other oysters collected at the same time and place. Such oysters may exhibit either advanced or retarded gonad development. In one case, for example, a large number of oysters collected on March 20, 1939, in Narragansett Bay contained a female oyster whose gonad resembled the partly spawned condition which is usually observed in July. Apparently, spawning of this individual was delayed, and the animal entered the hibernating stage before discharging all of its spawn. It is unusual, however, that in that case there was no evidence of cytolysis or resorption of unspawned eggs, which in all respects appeared normal and healthy.

In another case of similar nature, a male oyster, with gonad filled with spermatozoa, was found in a sample of oysters taken from Long Island Sound in the middle of March, when the water temperature was near 0° C. When some of this gonad material was placed in a drop of sea water and examined under a microscope, the spermatozoa could be seen actively swimming in the surrounding medium. Histological examination showed that the content of the gonadal follicles consisted largely of ripe spermatozoa, whereas the cells of earlier stages of spermatogenesis were very few. This observation indicates that ripe spermatozoa found in the male were the product of the previous year's gametogenetic activities. Therefore, it is quite possible that in some rather rare and unusual cases an oyster may retain large quantities of ripe spermatozoa throughout the winter.

A wide difference in the degree of maturity of oysters taken from the same bed and at the same time has been reported by Nelson (1928) who conducted his observations in Barnegat Bay.

In collecting samples from the beds of Long Island Sound, an opportunity presented itself to compare the condition of the gonads of oysters living at different depths. As far as the beginning of spring gonad development was concerned, very little difference was observed between the groups of oysters brought from the depths ranging from mean low water mark to 40 feet. Although some of the oysters in each group were

in either advanced or retarded stage, the majority of oysters, regardless of the depth they came from, were in about the same stage. It was noted, nevertheless, that prior to the beginning of spawning the oysters of shallow water accumulated much larger quantities of spawn than the deep water oysters. Within the depth range studied the beginning of spawning occurred about the same time. However, the shallow water oysters usually completed spawning somewhat earlier than the animals living in deeper water.

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OBSERVATIONS ON THE DUAL CONTRACTION OF CRUSTACEAN MUSCLE[1]

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Crustacean skeletal muscle possesses characteristics of both smooth and striated muscles of vertebrates. Like smooth muscle, its inhibitory mechanism is peripheral rather than central (Richet, 1879). It does not respond so readily to single induction shocks as to repetitive stimulation. Under appropriate stimuli its contractions are sluggish, and maximal tonic contractions can be maintained for long periods without apparent fatigue (Richet, 1879). On the other hand, the muscle is cross-striated; is under voluntary control; and is capable of quick powerful contraction and prompt relaxation. As in vertebrate skeletal muscle, tonus, when present, is probably maintained through impulses passing to the muscle from its nerve (Barnes, 1930). The two types of response present such sharp contrasts that as a result of his studies on Astacus, Lucas (1917) concluded that the adductor muscle of the claw possesses two distinct neuro-muscular systems. He arrived at this conclusion mainly because of the break in the strength-duration curve of excitation associated with an abrupt change in the character of the muscular response. These two types of contraction so characteristic of crustacean muscle were the subjects of the present investigation.

Experimental

Experiments were made on the blue crab (*Callinectes sapsidus*), the spider crab (*Libinia canaliculata*), and the lobster (*Homarus*) under temperature conditions prevailing at the Woods Hole laboratory during the summer season. A claw-bearing appendage was removed and placed in a clamp. Stimuli were applied either to the nerve placed on non-polarizable electrodes or directly to the muscle by silver-silver chloride electrodes introduced through small openings in the shell. Induction shocks or condenser discharges were used to excite. Contractions of the adductor muscle were recorded either by attaching a lever directly

[1] Aided in part by a grant from the Hendricks' Research Fund, Syracuse University.

to the muscle tendon or to the movable jaw of the claw, the tendon of the adductor being cut.

Single Brief Electrical Currents

Proceeding as above, single shocks were applied at regular intervals and intensity gradually increased from a subliminal value. Intervals were of sufficient length so that cumulative summation of effects of previous stimuli did not occur. Under such conditions, the quick contraction (twitch) always appeared first. As stimulus intensity was increased, the slow type of contraction appeared as a shoulder broadening the curve (Fig. 1*A*). If the time interval between stimuli is shortened,

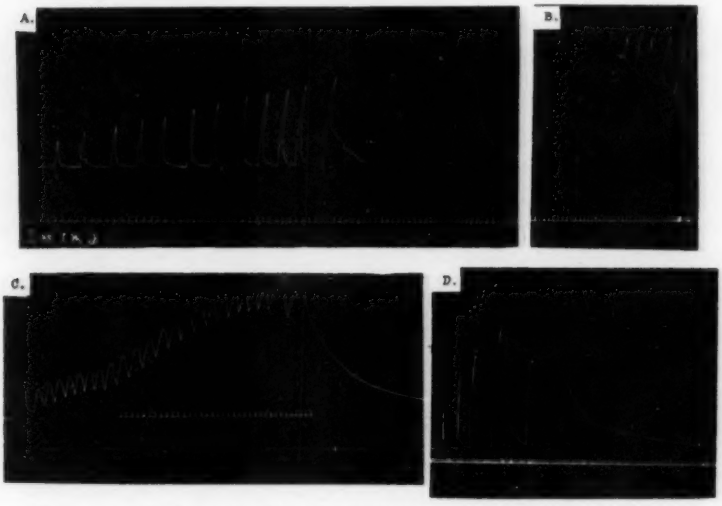

FIG. 1. *A*. Spider crab adductor stimulated directly by single condenser discharges of increasing E.M.F. Time in seconds.

B. Same preparation as *A*. Weaker stimuli applied during relaxation of slow contraction.

C. Spider crab adductor stimulated directly by single condenser discharges. Quick contractions are superimposed on a slowly rising tonus. Time in seconds.

D. Spider crab adductor stimulated through its nerve by single condenser discharges. Fourth and fifth contractions show a slow contraction developing after the quick contraction is over. Time in seconds.

summation effects appear and the quick contractions are added to a slowly rising tonus wave (Fig. 1*C*). When the muscle was stimulated directly by a single condenser discharge or an induction shock, the E.M.F. had

to be increased three or four fold before the slow contraction appeared. With further increase, the contraction became progressively prolonged and frequently lasted several minutes following a single strong stimulus. By a slow repetition of the stimulus, e.g., 1 per second, the contraction under light tension could be continued for more than 10 minutes without evidence of fatigue. During the relaxation of the slow contraction, twitches could be superimposed by applying appropriate weaker stimuli (Fig. 1*B*). Results were essentially the same whether the stimulus was applied to the nerve or directly to the muscle, except that the nerve rapidly lost excitability under strong stimulation and only responded briefly to the higher intensities used. At the prevailing room temperature (23° C.), the total time of the quick twitch was not especially different from that of amphibian muscle, being in the neighborhood of 0.1 second for *Callinectes* and 0.2 second for *Libinia*. The duration of the slow contraction was a matter of seconds. With strong stimulus, it continued to develop after the quick component was over (Fig. 1*D*).

Repetitive Stimuli

Repetitive stimulation introduces the possibility that various factors, such as summation, fatigue, refractory or supernormal phases, may modify the result. To give repetitive stimuli, a motor-driven rotating double key was arranged to act as a charge-discharge key for condensers or to deliver make or break induction shocks as desired. The rate of stimulation was controlled by motor speed and reducing gears. By this means stimuli of varying frequency and intensity could be applied as desired. The determination of threshold, by this method, applying repetitive stimulation for brief periods, reversed the results obtained with single shocks. At a frequency below a certain limit any effects observed were those of single stimuli but if an intensity was chosen which was subliminal for the single stimulus and applied repetitively, the slow contractions appeared at the lower rate (Fig. 2*C, D*) to change to the quick type as frequency or intensity was increased. This change from slow to quick contraction was an abrupt one and not a gradual shortening of the contraction time. The frequency at which the abrupt changes appeared varied with the intensity of the stimulus and the condition of the preparation. This is in agreement with the results obtained on other crustacea by Blashko, Cattell, and Kahn (1931), and by Pantin (1936). Changing the procedure, the intensity of the stimulus was varied at different fixed frequencies. The results were essentially the same as above, i.e., the slow contraction appeared at the lower threshold (Fig. 2*E*). At very low frequencies a limit was reached where, if intensity was sufficient,

effects were those of single stimuli and quick contractions appeared either singly or as saw-toothed notching on a slowly rising tonus contraction (Fig. 2*A*). With frequencies around 10 per second or higher, the slow contraction always appeared first as intensity was increased from subliminal values and changed to the quick type with further increase of intensity. On slow contractions as they developed or relaxed, quick contractions could be superimposed by application of single stimuli of proper intensity or by brief repetitive stimuli of greater strength or frequency.

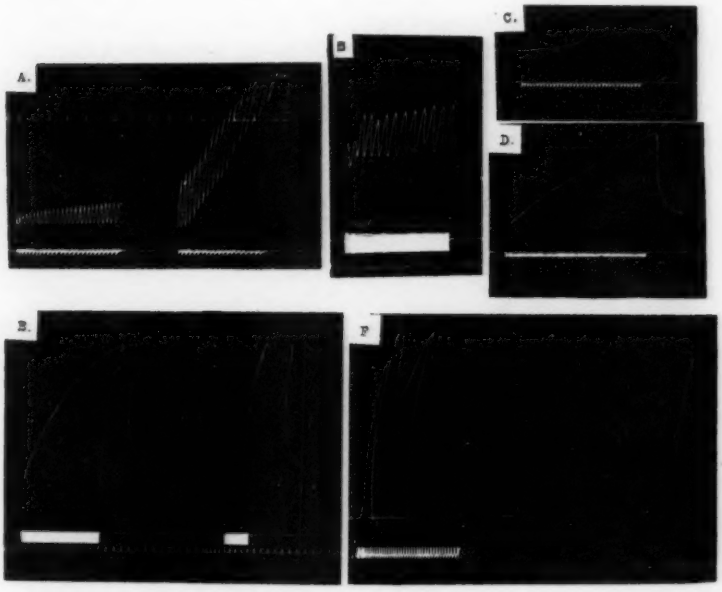

FIG. 2. *A*. Blue crab adductor stimulated by increasing intensity of repetitive induced currents (Harvard induction coil). Signal indicates stimulation frequency (4 to 6 per sec.). Time = sec.

B. Rhythmic quick contractions of lobster adductor stimulated directly by weak tetanizing current (vibrator). Time = sec.

C. Spider crab adductor stimulated at rate of 11 per sec. The slight irregularities are due to variations in output of stimulator. Signal indicates stimulation frequency. Time = sec.

D. Same preparation as *C*. Stimulation rate 14 per sec. Time = sec.

E. Blue crab adductor stimulated repetitively (vibrator) through its nerve with secondary induction of coil at 8 cm. and 6 cm. Lines drawn from crests of the curves indicate differences in time of slow and quick contractions. Time = 1/5 sec.

F. Blue crab preparation as in *E* shows the development of quick and slow contractions in the same curve. Strong stimulus. Time = 1/5 sec.

An especially interesting phase of response to repetitive stimulation was the frequent appearance of rhythmic twitches superimposed on the slow contraction. This rhythm when it appeared was quite independent of the frequency of stimulation. These rhythmic contractions appeared at stimulation intensities near the threshold and they often persisted with quite uniform rhythm during the period of stimulation (Fig. 2*B*). It was suggested by Knowlton and Campbell (1929) that they may be due to simultaneous excitation of inhibitory fibers or nerve endings.

Discussion

These experiments show that either the slow or the quick contraction may be excited selectively or both types may be excited simultaneously. When they are excited simultaneously the function of the slow contraction is to maintain the tension developed by the quick contraction. Bronk (1932) has presented evidence that it is the function of the slow contraction to maintain tension with less expenditure of energy associated with a slower rate of nerve discharge. As noted above, this tension can be sustained for a long time without evident fatigue.

With single stimuli of moderately short duration, such as induction shocks or condenser discharges, the quick contraction is elicited at a much lower threshold. With repetitive stimulation it requires greater frequency or intensity. Time relations are not unlike those of the twitch of amphibian muscle. On the other hand, in contrast to these characteristics, the slow contraction with its longer chronaxie or excitation time, has a much higher threshold for single stimuli, except for constant currents of long duration (Lucas, 1917). It is excited by repetitive stimuli of either lower frequency or intensity. With increasing strength of stimulation above the threshold, the contraction becomes more and more prolonged and may persist for some minutes after the stimulus has ceased.

The two types of contraction exhibited by crustacean muscle are so divergent in their characteristics that they suggest that they may be due to different muscle fibers. This was the conclusion arrived at by Lucas (loc. cit.). Indeed this possibility has not been entirely excluded. However no corresponding differences in the fibers of a given muscle have been observed, nor have differences in the innervation of the fibers composing any single muscle been described. It seems more probable that each fiber is capable of responding differently as frequency and possibly other charasteristics of the excitatory nerve impulses differ. In the evolution of muscle, smooth muscle with its sluggish contraction appears first. With the development of cross striation, ability to rapidly contract and relax appears. More primitive striated muscle like that of crustacea

normally exhibits both types of contraction. In normal skeletal muscles of certain vertebrates such as those of the frog (*Rana temporaria*), the slow contraction (contracture) may still be elicited by appropriate stimuli (Bremer, 1932). Bremer's records of contracture in amphibian muscle find remarkable parallels in the records from crustacea presented in this paper. Such contractures are not the operation of a special contractile mechanism but the expression of a specific type of excitation of the muscle fibers (Bremer). Methods of excitation are generally similar to those causing slow contraction of crustacean muscle. In mammals no contracture has been shown to function in the normal movements of the body though contractures may appear under abnormal and pathological conditions as in certain myopathies or following the section and subsequent degeneration of a motor nerve. These contractures have been reviewed in detail by Gasser (1930). Thus slow contraction is found in all types of striated skeletal muscle. It is a normal mechanism in crustacea but vestigial in mammals. Its specific type of excitation is characterized by the slowness of its development and disappearance. Its long chronaxie requires a single stimulus of long duration (Lucas, 1917) or excessive strength or stimuli of lower intensity or duration applied repetitively so that they produce their effect by summation.

It seems entirely probable that the single stimulus of high intensity acts by setting up a repetitive effect in nerve fibers or nerve terminals. It is not possible to eliminate such effects by curare, since motor endings in crustacea are not paralyzed by curare (author's observation). Verzar and Ludany (1929) observed rhythmic electrical discharges from passage of a constant current through crustacean nerve. Barnes (1930) also noted that a repetitive nerve discharge was set up by strong stimulation as cutting or crushing, and Van Harreveld and Wiersma (1936) recorded repetitive action currents in the nerve associated with the slow contraction. Whatever its nature, the excitation process resulting in the slow contraction is characterized by remarkable powers of summation. Stimulation, even if subliminal, sets up persistent changes which are additive. Contraction may appear only after 40 or 50 separate stimuli extending over a number of seconds. It is this persistence of effects and their consequent summation which explains the selective effectiveness of repetitive stimulation for excitation of the slow contraction. On the basis of evidence at hand, it seems probable that the slow contraction of crustacean muscle can occur only as the result of stimuli which are repeated and summed.

The slow development and disappearance of this additive excitatory effect make it intriguing to consider the possibility that a humoral mechanism is involved. Many of its characteristics, especially its time rela-

tions, could be satisfactorily explained on this basis. Among the known humoral agents of vertebrates, adrenalin causes contraction of crustacean muscle (Du Buy, 1935). Pituitrin (vasopressin) also causes contraction and these contractions are of the slow type (author's observation).

As different frequencies selectively excite different types of contraction, these frequencies could be transmitted by one nerve fiber and it is not necessary to postulate multiple excitatory innervation though this possibility is not excluded. In a series of papers, Van Harreveld and Wiersma (1936–1939) have presented evidence that innervation varies in different crustacea. Examples of single, dual, triple, and even quintuple innervation are described. Even the different muscles of the same individual differ in mode of innervation (Van Harreveld 1939). Multiple innervation furnishing exclusive paths for different activities appears to possess distinct advantages over a single common path though it is not a necessity nor is it uniformly present.

Summary

The slow and quick types of contraction of skeletal muscle of certain crustacea have been investigated and characteristics of the two types of contraction are described. These contractions are correlated with "twitch" and "contracture" of vertebrate muscle.

By appropriate stimuli, one may excite either type of contraction selectively or may excite both simultaneously. When both types are produced by a single strong stimulus, the slow contraction functions to sustain and continue the tension developed by the quick contraction.

Due to the remarkable powers of summation associated with excitation of the slow contraction, it is most easily excited by repetitive stimuli of low intensity. Possibilities of a humoral factor are suggested.

On such a slow contraction, quick contractions may be superimposed by sudden increase of frequency or intensity.

Thus by appropriate stimuli one may excite either type of contraction selectively or may excite both simultaneously.

The function of multiple innervation is discussed briefly.

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WING FORM AND GENE FUNCTION IN NINE GENOTYPES OF DROSOPHILA MELANOGASTER [1]

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We have reported previously on a number of experiments on the vestigial locus of *D. melanogaster* in which marked changes of wing form were studied (Harnly, 1930*a*, *b*, 1936, 1940*a*, *b*, 1941; Harnly and Harnly, 1935, 1936). During the course of those experiments a number of minor wing margin defects were observed at certain temperatures in otherwise constant normal phenotypes. Other genotypes, approaching the wild phenotype in only one part of the viable temperature range, had major or minor wing margin variations in those nearly normal wings. A larval thermolabile period in wing development at high temperatures for the mutant vestigial of *D. melanogaster* has been reported by several workers (Harnly, 1930*b*, 1936; Li, 1936; Stanley, 1931, 1935). The pupal period of vestigial has proved refractory at high temperatures. The following experiments were: (*a*) an attempt to find a pupal thermolabile period in vestigial; (*b*) a study of the effects of less extreme alleles and modifiers on wing margin variation; and (*c*) the determination of the temperature-effective-period of these wing margin variations in preparation for an histological study of wing development during the larval and pupal periods.

Technique

Unless otherwise specified, the stocks used in these experiments have all been backcrossed in single pair matings for many generations to males from our inbred vestigial stock (single pair sib matings). These strains should therefore differ only by the mutant genes indicated. Mass matings of the genotypes examined laid eggs for a one or two hour interval on small trays of banana-agar media painted with a heavy yeast suspension. These trays were incubated at 25° C. for 24 hours. Ten recently hatched young larvae were then transferred to each 1 × 4 inch vial containing the yeasted customary banana-one per cent agar medium. This

[1] Aided by a grant from the Penrose Fund of the American Philosophical Society of Philadelphia.

concentration of larvae per unit area is well below the point where crowding affects the wings. The larvae transferred were selected for uniform size. This technique insures uniformity of larval age as any eggs that have been held back by the females after fertilization will have hatched earlier and all these larger larvae were discarded. The high degree of accuracy of temperature control in our incubators has been discussed previously (Harnly, 1936). Vials of larvae transferred from a high to a low temperature (16°) were placed first at 10° for thirty minutes to lower rapidly the temperature of the food mass. The treatment of the larvae is described below for each genotype. The wings of all emerged flies were examined under 15 × magnification.

Thermolabile Period for Vestigial, Dimorphos Vestigial, and Wing-Margin Genes

One hundred and forty vials of newly hatched vestigial larvae were placed at 32° until they had completed a total of 120 hours of development from the time the eggs were laid. Seventy vials were then transferred to 25° and the other seventy vials to 16° for the completion of development. The wing size and phenotype ("notched") of the flies emerging at 25° and at 16° were the same and similar to those previously reported for transfers at this time interval from 32° to 25° (see Harnly, 1936 for wing drawings and measurements for 32° to 25°).

Vials containing newly hatched vestigial larvae were placed in 32° incubators. Beginning shortly before the onset of puparium formation these vials were examined hourly and recently formed puparia isolated. One-fourth of these were immediately placed at 25° for the completion of development and emergence. The second quarter were placed at 16° for all subsequent development. The third quarter remained at 32° for an additional 24 hours and were then placed at 16°. The fourth group were transferred to 16° where they remained for six days and were then transferred to 25° for emergence. The results in all four groups were identical with each other and with those described above. There was only one significant difference between those transferred at 120 hours and at puparium formation. In the latter animals the wings were much more frequently bilaterally symmetrical in size and general form.

Pupation is extremely difficult at 32° but occurs normally at 28°, 11 hours ± 30 minutes after puparium formation. Vials containing recently hatched vestigial larvae were placed in 32° incubators. At the time of puparium formation regular inspections were made and puparia formed within the hour were isolated and placed in 28° incubators. The controls remained at 28° for the completion of development and the

emergence of the imagos. After 12 hours at 28° the rest of the vials were returned to 32° where one third remained for 12 hours, one third for 18 hours, and one third for 24 hours. All of these pupae were returned to 28° for the completion of development because homozygous vestigial flies do not emerge at 32°. The results obtained with these three sets of experimental animals were identical with their controls, with those obtained for transfers in this work from 32° to 25° and 16°, and with those previously reported for transfers from 32° to 25° for similar time intervals (Harnly, 1936).

We have reported previously on temperature-effective-period tests of the reciprocal type (Harnly, 1930*b*). In those experiments the development of the vestigial animals began at 29°, one degree below the critical temperature for the increase in wing size (Harnly, 1930*a*). The controls remained at 29° for total development. Groups of experimental animals were transferred at intervals of 24, 48, and 72 hours after egg laying, puparium formation, pupation, and 24 and 48 hours after pupation from 29° to 31° for the completion of development and the emergence of the imagos. Again the thermolabile period ended at the time of puparium formation. From all the experiments reported above it is evident that in the case of the vestigial genotype there is no thermolabile period for the development of wing form during the pupal stage at high, intermediate, or low temperatures.

All of the varied experiments on the vestigial genotype reported above were repeated with the dimorphos vestigial genotype. (Dimorphos is a sex-linked recessive modifier of vestigial.) No temperature response occurred during the pupal period either at high, intermediate, or low temperatures, hence the temperature-effective-period is restricted to the larval stage in this genotype. (For a description of dimorphos vestigial and its general response to temperature see Harnly and Harnly, 1935.)

In previous work with the stock of vestigial-pennant (a reverse mutation from vestigial to a recessive "wild") that had been repeatedly back-crossed to the inbred vestigial strain perfect wild-type wings were produced at 16° on part of the flies; animals developing at all higher temperatures had minor nicks on both wings (Harnly and Harnly, 1936).

Vials of newly hatched larvae were placed at 25° to determine whether this nicking is thermolabile prior to or after puparium formation. Control vials remained at 25° for the completion of development and emergence of the adults. Experimental animals were transferred to 16° at 96 hours after egg laying and at the hour of puparium formation which took place 110–130 hours from egg laying, the bulk of the puparia being formed between 114 and 122 hours. There were no perfect wings on 477 control males and 445 control females nor on 708 males and 552 females

transferred to 16° the hour they had formed puparia. The 96-hour shift to 16° gave 17 per cent of the males with one perfect wing and 3 per cent of them had both wings perfect; 15 per cent of the females had one perfect wing and an additional 6 per cent had both wings perfect. The data demonstrate that the presence or absence of minor nicks in this genotype is determined by the temperature experienced during the larval period prior to puparium formation. Since the frequency of normal wings was not as high as that obtained for total development at 16° the temperature-

TABLE I

Wing characteristics of stock b vg^p, reared at temperature indicated, 2 trials. O—number of flies neither wing showing characteristic indicated, L—left wing shows, R—right wing shows, LR—both wings show

°C.	Wing Variation	Males				Females			
		O	L	R	LR	O	L	R	LR
16	Margin Normal				340				310
25		38	68	69	162	236	47	39	45
30		124	87	88	75	181	42	27	12
30	Margin Normal –V		7	16			16	10	1
25	Extra X-Vein II–III	206	50	49	32	263	51	44	9
30	Extra X-Vein II–III	230	51	59	34	209	16	23	14
	Extra X-Vein III–IV	340	10	23	1	237	14	11	
	Extra X-Vein II–III and III–IV	365	5	3	1	256	2	3	1
16	Delta	258	35	37	8	219	35	41	15

effective-period must begin prior to 96 hours of total development and end after 96 hours but no later than the time the puparia are formed.

WING-MARGIN AND VEIN GENES

It had been observed that the original black vestigial-pennant stock threw a good many males and females with normal wing margins at 25°. Black vestigial-pennant animals were reared at 16°, 25°, and 30°. The data are shown in Table I. They differ markedly from those obtained from the backcrossed vestigial-pennant flies. Both wings of all black vestigial-pennant flies had normal margins at 16° and a fairly large

proportion of the flies had at least one wing with a normal margin at 25° and 30°. The difference in the results with the two vestigial-pennant stocks indicated that the marginal nicks must be due, at least in part, to one or more other loci.

Green and Oliver (1941) have reported "that a larval temperature of 27° is sufficient to increase significantly the wing length of black-vestigial and yellow-vestigial males and females. . . . No such effect was noted among vestigial and vestigial-sooty individuals." The differences between the wings in comparative samples of these genotypes were not only significant but actually profound. We have tested the black vestigial and the yellow vestigial stocks carried in our laboratory at 25°, 27°, 28°, 29°, 30°, 31°, and 32°. All of the males and the females reared at the intervals from 25° through 29° had typical "vestigial" wings. Our previously reported changes in phenotype at 30° and above were also observed in these two stocks. Evidently, these pigment genes do not influence the phenotype of homozygous vestigial and Green and Oliver are dealing with some other modifier of vestigial lowering markedly its critical temperature as does dimorphos (Harnly and Harnly, 1935). Mr. Green informs me that in view of certain internal evidence in his stocks, this interpretation is probably correct.

Four new conditions were observed in the wings of the black vestigial-pennant stock. (1) The distal end of the V-longitudinal vein was absent at 30° in 8–10 per cent of the wings with perfect margins. (2) An extra cross-vein between the II and III-longitudinal veins proximal to and near the anterior cross-vein was present at 25° in at least one wing on 40 per cent of the males and 28 per cent of the females. At 30° this extra cross-vein appeared on the wings of 40 per cent of the males and 20 per cent of the females. (3) An extra cross-vein between the III and IV-longitudinal veins was seen at 30° on 9 per cent of the males and the females; and an occasional animal had both extra cross-veins on the same wing. There was no obvious relation between a perfect or imperfect wing margin and the appearance of these extra cross-veins. (4) A delta appeared at the junction of the posterior cross-vein with the IV-longitudinal vein in at least one wing of approximately 25 per cent of the males and the females at 16°.

Temperature-Effective-Periods of Margin Nicks, Extra Cross-Veins, Delta, and Incomplete V-Vein

Transfers were made from 30° to 16° to determine the period during development when these conditions are thermolabile. The results are

shown in Table II. The data indicate that the temperature-effective-period for marginal nicks (all animals normal at 16°) begins at approximately 60 hours in both sexes and ends for the females at about 90 hours and for the males around 96 hours of total development. Mr. Herbert J. Levin (unpublished) has found that the mean time from egg laying of the molt between the second and the third instar in our inbred vestigial stock is 68 hours at 25° and 59 hours at 31°. Evidently the thermolabile period for marginal nicks in the black vestigial-pennant stock begins with the molt from the second to the third instar and terminates before puparium formation. The temperature-effective-period for both types of extra cross-veins (appear at 30°) must be during the pupal period since these veins were not found in the wings of animals transferred from 30° to 16° earlier than 120 hours. The delta (shows at 16°) appeared with

TABLE II

Stock b vg^{p}, frequency of flies with normal wing margins, animals developing at 30° are transferred to 16° at the intervals indicated, 2 trials.
Legend O, L, R, LR as in Table I

Wing Variation	Age at Transfer	Males				Females			
		O	L	R	LR	O	L	R	LR
Margin Normal	60 Hours		1		123		3	4	207
	72 Hours		3	3	242	5	26	40	278
	78 Hours	2	3	5	148	30	47	34	165
	84 Hours	3	8	14	70	64	20	24	66
	90 Hours	13	11	16	14	93	19	19	7
	96 Hours	21	11	11	19	111	19	16	24
	120 Hours	19	36	28	62	136	34	38	20

the usual frequency in the groups of flies from all of these time intervals and would seem to be determined during the pupal period.

The reciprocal transfers from 16° to 30° of individuals isolated at the hour of puparium formation constitutes a critical test of the above interpretations. Both wings had normal margins on all the males and the females transferred from 16° to 30° immediately after puparium formation. Furthermore, some 60 per cent of the males and 54 per cent of the females had the extra cross-vein between the II and III-longitudinal veins on one or both wings. Twenty per cent of the males and 22 per cent of the females had an extra cross-vein between the III and IV-longitudinal veins on one or both wings. Both extra cross-veins were observed in the same wing on one or both wings of 41 per cent of the males and 34 per cent of the females. All possible combinations of these

three conditions were seen on the two wings of different individuals. These reciprocal transfers demonstrate conclusively that the entire temperature-effective-period for the presence or absence of the wing margin nicks is during the third larval instar prior to puparium formation and that the critical period for the extra cross-veins is restricted to the pupal stage. The delta (appears only at 16°) did not show in the wings of any of these animals and therefore has its thermolabile stage restricted to the period following puparium formation. Even with complete development at 30° the frequency of the incomplete V-longitudinal veins is low. Consequently the data from the reciprocal transfers is inconclusive but suggests that the temperature-effective-period for this character follows puparium formation.

Black Vestigial-Pennant/Vestigial (Unrelated)

The marked difference between the data of the vestigial-pennant (derived from repeated backcrossing to inbred vestigial) and the black vestigial-pennant stocks on wing margin nicks, delta, extra cross-veins, and V-longitudinal vein, indicate that these two stocks of vestigial-pennant must differ by one or more other wing determining genes. Experiments were performed to determine something of the number and nature of these other wing genes.

We have shown previously that the heterozygote between the inbred vestigial stock and the vestigial-pennant stock that had been repeatedly backcrossed to it produced deeply notched wings at 32° and "cut," "antlered," and "strap" wings at lower temperatures (Harnly and Harnly, 1936). The original black vestigial-pennant stock was crossed to a non-inbred vestigial stock. This heterozygote produced rather large numbers of males and females at 32° with both wings having perfect margins. The flies with imperfect wing margins at 32° had only very minor nicks in the distal end of the wings between the III and IV-longitudinal veins. Extra cross-veins appeared fairly frequently in the wings of the flies reared at 32°. At lower temperatures the wings were markedly defective and similar to those reported previously (Harnly and Harnly, 1936). The difference in the wings produced at 32° by these two identical vestigial locus heterozygotes must be due to differences in other loci; one set of modifiers producing deeply "notched" wings, the other perfect wild-type wings.

Dominant-Recessive Determinations

Reciprocal crosses were made between the vestigial-pennant (derived from repeated backcrossing to inbred vestigial) and the black vestigial-pennant stocks to determine whether the modifiers causing the delta, extra

cross-veins, an incomplete V-longitudinal, and margin nicks are dominant or recessive, sex-linked or autosomal. The offspring were reared at 16°, 25°, and 30°. The results are shown in Table III.

TABLE III

Variation of wing characteristics, F_1 of reciprocal crosses reared at temperature indicated, 2 trials. Legend O, L, R, LR as in Table I

	Wing Variation	°C.	Males				Females			
			O	L	R	LR	O	L	R	LR
b vg^p ♂ × b$^+$ vg^p ♀	Margin Normal	16	1	16	13	129	2	18	15	153
		25	116	11	18	3	103	26	16	6
		30	56	72	72	112	93	32	28	205
	Delta	16		3	1			12	6	1
	Extra X-Vein III–IV	30							1	
b$^+$ vg^p ♂ × b vg^p ♀	Margin Normal	16	2	7	7	170	2	9	8	186
		25	59	34	42	31	78	33	26	10
		30	5	45	43	396	36	33	31	344
	Delta	16		1	1			9	6	
	Extra X-Vein III–IV	30		3	3				3	

An extra cross-vein between the II- and III-longitudinal veins in one or both wings was found frequently on the black vestigial-pennant males and the females at 25° and 30° (Table I). This extra cross-vein was not observed on any of the offspring from the reciprocal crosses at either 25° or 30°. The extra cross-vein between the II- and III-longitudinal veins is evidently due to an autosomal recessive.

An extra cross-vein between the III- and IV-longitudinal veins was observed on some of the black vestigial-pennant males and females reared at 30° (Table I). The data from the reciprocal crosses seems to indicate that this extra cross-vein is controlled in part by a semi-dominant sex-linked gene carried in the X-chromosome of the black vestigial-pennant stock. However, its expression must be modified by an autosomal gene in the backcrossed vestigial-pennant stock since the frequency in the sons out of black vestigial-pennant by vestigial-pennant is markedly reduced.

The evidence is inconclusive on the mode of inheritance and dominance of the gene occasionally causing an incomplete V-longitudinal vein at 30°.

The delta appears with a lower frequency in both sexes among the offspring of the reciprocal crosses and is probably due to an autosomal semidominant not finding expression as frequently in the heterozygote as in the homozygote. In the homozygote it appears significantly more frequently in the females than in the males. In the heterozygote this sexual dimorphism in frequency is profoundly increased.

Clear-cut conclusions cannot be drawn on the mode of inheritance of the margin gene or genes. The data of the males seem to indicate that the X-chromosome of the black vestigial-pennant stock carries a gene tending toward normal margins. This is indicated by the higher percentage of males with normal margins from black vestigial-pennant mothers than the reciprocal males with vestigial-pennant mothers. However, the sisters of the first type also have a higher frequency of normal wings than the reciprocal females. These two sets of females should have identical genotypes. Likewise the change in frequency with temperature is disconcerting. A fair percentage of the vestigial-pennant (derived from repeated backcrossing to inbred vestigial) flies have normal wings at 16° but produce no normal wings at higher temperatures. The black vestigial-pennant stock produces only normal wings at 16° and at higher temperatures produces a steadily decreasing proportion of normal wing margins. The reciprocal heterozygotes produce the lowest percentage of normal margins at 25° with markedly higher percentages of normals at both 16° and 30°. Since the two homozygotes have similar temperature responses, a shift in dominance with temperature in the heterozygote corresponding to that found in vestigial/vestigial-pennant (Harnly and Harnly, 1936) does not seem possible, i.e., this difference cannot be due to a change having taken place in the vestigial-pennant gene in one of these two stocks. Probably more than one locus, other than the vestigial locus, is concerned in the production of marginal nicks. It may be that these genes are the domini genes of Goldschmidt but it would take additional work to determine this point (Goldschmidt, 1937*a*, *b;* Goldschmidt and Höner, 1937).

Proof Other Loci Cause Differences Between Vestigial-Pennant Stocks

A critical proof that the differences between these two vestigial-pennant stocks (extra cross-veins, degree of margin nicking, delta, etc.) were not due to the vestigial locus would come from a transfer of these differences from one stock to another stock. The black vestigial-pennant line was crossed to an unrelated vestigial strain not used in any of our previous work. From the F_1 hybrids two new stocks were developed.

In the first case black vestigial-pennant/vestigial females were backcrossed to black vestigial-pennant males and through forty generations the hybrid females were backcrossed to the original black vestigial-pennant strain (single pair matings). Homozygous vestigial was then obtained by inbreeding the forty first generation. This new vestigial line should carry the I, III, and IV chromosomes and through cross-overs practically the entire II-chromosome of the black vestigial-pennant stock, differing from it only at the vestigial locus and perhaps a few adjacent loci. In the second case the F_1 hybrid females and their daughters through forty generations were backcrossed to males from the unrelated vestigial stock (single pair matings). Inbreeding the forty first generation produced a new vestigial-pennant line which likewise should have had the genes replaced at all loci save the vestigial locus. These two new stocks were then tested.

Larvae of the new vestigial stock were reared at 32° until puparium formation and then transferred to 30° for the completion of development and emergence. A very high percentage of these flies had an extra cross-vein between the II- and III or III- and IV-longitudinal veins (or both) on one or both wings. The presence or absence of these extra cross-veins is not dependent on the gene at the vestigial locus. They are therefore due to other independent loci. The wing phenotype and size were similar to that reported previously for vestigial reared at 32° (Harnly, 1936). The only significant difference observed in wing shape and size was a tendency toward more nearly perfect wings. An occasional fly had only minor nicks or a small notch in the wing at the distal end between the III- and IV-longitudinal veins. This would indicate that the gene or genes for less nicking (normal margins) in the black vestigial-pennant stock had been transferred to the vestigial stock by the repeated backcrosses. It furthermore emphasizes the fact that the size and form of the wing are determined by temperature, the vestigial gene, and other loci as well; in other words, by the entire genotype *and* the environment. The delta at the junction of the posterior cross-vein with the IV-longitudinal vein reported above appears only in the wings of flies reared at 16°. Due to the small size of the vestigial wings at this temperature, it was impossible to test for the presence of this gene.

The new vestigial-pennant stock was tested for the replacement, by backcrossing, of the assumed genes. The data demonstrate that this substitution had taken place (Table IV). Some of these flies differed from the black vestigial-pennant stock by having defective wing margins at 16° but the percentage of flies with one or two normal wings was double that produced at 16° by the vestigial-pennant stock derived from repeated backcrosses to our inbred vestigial line. No perfect wings were

produced at 25°; but at 30° some wings with normal margins were produced. Here again the data differ from that obtained from the other backcrossed vestigial-pennant strain which produced normal wings only at 16° (Harnly and Harnly, 1936). At all three temperatures the data on the wing margin differ from that of the black vestigial-pennant stock (Table I) from which it had been derived by repeated backcrosses to the unrelated vestigial stock. Furthermore, the V-longitudinal vein was complete in all cases, the delta described for the black vestigial-pennant stock was not produced at 16°, and extra cross-veins did not appear at 25° and 30°. A different delta appeared at the junction of the posterior cross-vein with the V-longitudinal vein in the wings of some 14 per cent of the new type vestigial-pennant flies at 16°.

The data from the new vestigial and vestigial-pennant stocks demonstrate that: (1) the frequency of wings with normal or defective margins; (2) the presence or absence of a delta in the posterior cross-vein, and its location; (3) a complete or incomplete V-longitudinal vein; and (4) the presence or absence of extra cross-veins is due to genes independent from the vestigial locus.

TABLE IV

Frequency of wings with perfect margins, vg^p F_{40} strain, reared at temperature indicated, 2 trials. Legend O, L, R, LR as in Table I

°C.	Males				Females			
	O	L	R	LR	O	L	R	LR
16	21	37	47	222	22	48	50	319
25	357				418			
30	433	4	4		263	25	28	2

DISCUSSION

In these experiments nine genotypes have been subjected to the rigidly controlled environmental variable, temperature. The characters examined were: (1) wing form variation from "vestigial," "strap," and "antlered" through "notched" and, in some cases, "nicked" to "normal" in four genotypes; (2) wing form normal with and without marginal nicks in six genotypes; (3) the presence or absence of extra cross-veins at two unusual points in the wings of four genotypes; (4) the presence or absence of a delta in the posterior cross-vein at either its junction with the IV- or V-longitudinal vein in three genotypes; and (5) an incomplete V-longitudinal vein in two genotypes. The phenotype of each genotype

varied with the temperature experienced during development. When several genotypes had a common character variation the phenotype found at one temperature was characteristic of a specific genotype and was produced at a different temperature by another genotype. In previous papers (Harnly, 1930*a*, *b*, 1936, 1940; Harnly and Harnly, 1935, 1936) we have reported on a similar temperature response of genes affecting the wing form, the arrangement of the postscutellar bristles, size and form of the balancers or halters, arrangement of the ommatidia, size and form of the eyes, and the presence or absence of wing margin nicks. It is evident that the gene or even the whole genotype does not solely determine the phenotype of the individual.

The genotype of the individual is fixed at fertilization. However, this does not determine the phenotype of the individual. At this moment the limits within which each character may be expressed are set by the genotype of the particular organism. For example, in the dimorphos vestigial stock the wings will never be smaller than "vestigial" and may be as perfect as "normal," the balancers may be minute vestiges or normal in size and form, the postscutellar bristles may point anteriorly or posteriorly, and the eyes may be "normal" or markedly abnormal. The exact expression within these limits is determined separately for each character by the environment within which each individual develops. For example, in dimorphos vestigial the wings, postscutellar bristle, and the eyes change phenotype at different critical temperatures (Harnly and Harnly, 1935). Among the environmental factors that have been demonstrated to act as determiners within the boundaries set by the genotype are: temperature density of population, type or kind of food media, humidity, etc., for *D. melanogaster*. These and other environmental factors have been found acting in other forms of animals and plants. The subjection of the individual successively to two or more points within the environmental variable during the character's critical stage (thermolabile period) results in a phenotype characteristic of neither environmental point. In the case of vestigial the phenotype is an intermediate, the exact expression depending upon the duration of exposure to the two temperatures in the critical period (Harnly, 1936, Fig. 7). The data presented here, in our earlier papers, and in the work of others demonstrates that the environment is affecting the response or action of the genotype in a critical period of some duration during the development of each character. The limits (extremes) for each character of the individual are fixed at fertilization by the genotype established and the phenotype of each individual is *developed* at a particular point within those limits as the resultant of the interacting forces of genotype and environment during the critical stages of ontogeny.

During the course of these experiments a marked difference was observed between several stocks in the frequency of wings with marginal nicks, and previously undetected wing mutants were seen in the original vestigial-pennant stock when it developed at extreme temperatures. The genes for extra cross-veins, delta, and an incomplete V-longitudinal vein were replaced with their normal alleles by repeated backcrossing of the original vestigial-pennant stock to an unrelated vestigial stock. This replacement also changed markedly the frequencies of "normal" vs. "nicked" wing. The reciprocal repeated backcrossing of the unrelated vestigial stock to the original vestigial-pennant line transferred the genes for extra cross-veins to the vestigial strain and, through the shift in what one may call "wing-margin" genes, changed the degree of defect in the wings of vestigial flies reared at a high temperature. Presumably the gene for the delta was also transferred to the vestigial line but due to the form of the wings of vestigial flies reared at 16° it was impossible to test for the presence of the delta gene. The reciprocal tests demonstrated that the two differently located extra cross-veins, the delta, and the frequency of "normal" vs. "nicked" wing margins are all due to a number of different loci and independent in their expression of the particular allele at the vestigial locus. The data presented here and in previous papers demonstrate that the various isolated vestigial stocks have accumulated different and undetected mutations affecting the wing margin, venation, etc. These effects can appear only when crosses transfer the mutant genes to other stocks having wings of normal form or when the vestigial strain happens to develop at an extreme temperature where the wing is more nearly complete and the new mutants can be expressed. Evolutionarily speaking the situation is analogous to a balanced lethal form in which recessive mutants may collect and never show until a rare cross-over occurs. Due to the action of the vestigial gene only the basal region of the wing is developed. Mutant genes affecting potential regions or structures of the wing, which are not developed by the vestigial flies at usual temperatures, may accumulate in the strain even though not expressed. Then an outcross or a single mutation at the vestigial locus will allow the entire group to be expressed suddenly at one moment in time. A similar situation in nature might explain some of the more abrupt and complex changes in evolution.

The loci used in these experiments fall into one or the other of two groups according to the character affected. (1) The alleles at the vestigial locus in the 2-chromosome, at the dimorphos locus in the 1-chromosome, and at the wing-margin loci all affect the amount and form of the wings present. The various combinations of alleles at the vestigial and dimorphos loci determine whether the degree of imperfection

in the wings shall vary from "vestigial" to "notched," or from "vestigial" to "nicked" or "normal," or from "strap" or "antlered" to "notched," "nicked," or "normal." The minor defects on these genotypes are all terminal defects of notching or nicking in the distal end of the wing. The particular combination of wing-margin alleles and the environment determine the range of variation from none to one or more "nicks" in the margins of wings that are otherwise "normal" or approaching normal. The nicks caused by these alleles may appear at any point in the wing margin. (2) The other loci reported here had no detected effect on the amount and form of the wings present. Their effects were on the venation within the wings. An autosomal recessive determined the presence of an exceptional cross-vein between the II and III-longitudinal veins in certain environments; an extra cross-vein between the III and IV veins was similarly determined by an apparently semi-dominant sex-linked gene; the presence in the proper environment of an exceptional delta in the junction of the posterior cross-vein with the IV-longitudinal vein was caused probably by an autosomal semi-dominant, and a fourth locus was associated with the appearance of an incomplete V-vein.

However, there was no consistent temperature response through the viable range within either the wing form or wing venation group of loci and alleles. The vestigial gene for wing form responds directly to increases in temperature but its allele vestigial-pennant responds inversely through the viable range (Harnly, 1930*a*; Harnly and Harnly, 1936). The results of the reciprocal crosses between the two vestigial-pennant stocks (Table III) and those on the new vestigial-pennant stock (Table IV) demonstrate that some genes for wing-margin defects find expression at low temperatures while others find expression at high temperatures. The same heterogeneous response was found for the group of loci affecting wing venation. One of the genes associated with the extra cross-veins finds expression at both intermediate and high temperatures while the other one is expressed only at high temperature. The gene for an incomplete V-longitudinal vein is expressed only at a high temperature but the one for delta breaks through only at a low temperature. The nature of the response through the viable temperature range is not a function of the type of character or even of the locus. The nature of the response to temperature is a function of the particular allele.

But the stage in development when the gene is thermolabile is not a function of the particular allele or of the locus. Nor would one expect it to be so if the temperature-effective-period has any relation to ontogeny. All of the genes affecting wing form had a more or less common thermolabile period during the larval stage. The data presented here and previously (Harnly, 1930*b*, 1936; Li, 1936; Stanley, 1935) demonstrate

that the temperature-effective-period at low, intermediate, or high temperatures for the vestigial gene begins at or shortly following the molt from the second to the third larval instar and ends, depending upon the temperature and the modifiers present, during that instar or at its close when the puparium is formed. Similarly the data reported here for dimorphos vestigial demonstrate that its critical period is restricted to the third larval instar. Unpublished data from combinations of alleles at the vestigial locus also demonstrate that the thermolabile period for wing form never ends later than the time of puparium formation. The data reported here for two different combinations of wing-margin genes (nicks) shows conclusively that the temperature-effective-period for both genotypes is restricted to the third larval instar and ends by the time the puparium is formed. One may conclude that genes affecting wing form have their temperature-effective-periods during the larval stage and that this period is ended with the formation of the puparium. Our accumulated unpublished data on many genotypes demonstrate that wing size, in part or whole, is an entirely different problem from wing form and will be considered elsewhere.

The thermolabile period for the genes affecting wing venation is different from that of the genes associated with wing form. The genes at the two loci for extra cross-veins, the locus for the delta, and the locus for the incomplete V-vein were not affected in their expression by the temperature (low, intermediate, or high) at which the larvae developed. The temperature experienced following the formation of the puparium determined whether the exceptional mutant character or the "normal" expression was developed. Mr. Melvin Green has informed me that the temperature-effective-period of vesiculated (vs. 29c) is also during the pupal period of development (unpublished).

Each of these two groups of loci has its own characteristic thermolabile period. Hence, the temperature-effective-period for a character is not a function of the locus or the allele present. This interval must be a function or characteristic of a definite stage in development when the action of either a gene or its product is thermolabile. It seems that both the inception and maximum duration of this period are determined by or linked to general ontogenetic processes. Work now well along in our laboratory on the development of the dorsal mesothoracic discs of three genotypes shows profound morphological changes taking place in these discs and their wing buds during the third larval instar. In studies on post puparium development of a number of mutants affecting venation, Waddington (1939, 1940) has found that minor variation such as extra cross-veins, incomplete veins, and delta effects are determined or differentiated morphologically during his P_2 stage (18–45 hours) after pu-

parium formation. Both his findings on the morphological determination of the wing veins and our tentative conclusions on the morphological development of the dorsal mesothoracic discs and the wing buds would support the conclusion that the thermolabile period for a particular locus is the time of growth and/or differentiation of the structure affected by the gene. Either a precursor material is formed by the gene at an earlier stage and its subsequent action during ontogeny is thermolabile at a specific period morphologically determinable or the action of the gene modifying the development of the character is direct, immediate, and thermolabile at the moment of growth and differentiation of that character. In either case the genetic thermolabile period is a morphological stage of growth and differentiation of the character. The gene or its products cannot affect the development of a character until that stage is reached by the organism, e.g., molt to third instar for wing form, at which that structure is determined, grows, or differentiates. The developing structure then becomes responsive to the action of that gene or the gene products. Our earlier studies have shown that both the rate and the duration of this response is determined by both the genotype and the environment of the individual. If, due to the nature of the allele and the environment, the action is not self-terminated then it is automatically stopped by the attainment of a definite stage in development, e.g., in some genotypes puparium formation for wing form. During this period either the genes causing the minor modifications in the wing venation have not been functioning or the structure is refractory to them or their products. It is as if there was nothing or not enough there yet on which an effect could be made. Following puparium formation the development and differentiation of the wing has progressed to a stage where the final detailed differentiation of the veins can take place. It is not until this stage in ontogeny that these wing vein genes or their products can influence the structure. The thermolabile period is the experimentally determined stage in the development of a given character when the genotype under examination can cut in on and modify the developing structure. Comparative studies now in progress both within the single genotype and between genotypes of the morphological changes during this period should elucidate the effects of the gene upon the mechanics of wing development.

Summary

1. Nine genotypes, including a number of new mutants, affecting the wing form and the wing venation were found to vary in phenotype between 16° C. and 32° C.

2. The dominant-recessive relationships of these new mutants and the linkage group in some cases was determined.

3. The limits for the future phenotype of each character are demarked at fertilization by the genotype established. The phenotype of each individual is subsequently developed at a point within these extremes as the resultant of the interacting forces of the genotype and the individual's environment.

4. The nature of the response to temperature (positive or negative) throughout the viable range is a function of the particular allele.

5. All of the genes modifying the wing form were found to be thermolabile during the larval period prior to puparium formation.

6. All of the genes associated with minor modifications in the wing venation had their temperature-effective-period during the pupal stage following puparium formation.

7. The interval of the temperature-effective-period is not a function or characteristic of the allele or the locus but of the stage in development when the action of the gene or its products is thermolabile.

8. The rate of the response and the duration, if not the maximum, are determined by the allele and the environment.

9. The onset and maximum duration of this period are determined by definite developmental stages.

10. Apparently the development of the dorsal mesothoracic disc must progress to a certain point before the wing form loci become effective in further growth and differentiation of this structure and its wing bud. Loci causing minor modifications in the wing venation cannot become operative until much later when the final morphological differentiation of the wing is well advanced.

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SOME EFFECTS OF ANDROGENS ON THE ADULT MALE FUNDULUS

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Introduction

Concurrent with Matthews (1938, 1939*a*, 1939*b*), studies (Burger, 1939, 1940, 1941) have been made on the factors which influence the sexual cycle of the adult male *Fundulus heteroclitus*. These authors have found that warm water accelerates spermatogenesis in sexually inactive fish and that cold water retards the appearance of spermatogenesis and delays the testicular involution that follows the breeding period. Day-length has no effect on the testicular cycle. They have further found that *Fundulus* pituitaries implanted into hypophysectomized or normal males will stimulate spermatogenesis. The present study deals with the effects of two androgens,[1] principally testosterone propionate, on the sexual cycle of the adult male *Fundulus*. The literature contains few references to the effects of androgens upon the sexual cycles of adult fish. Pertinent references will be introduced in their proper context.

Materials and Methods

In all experiments freshly captured adult males, 7.5–9.5 gm., were used. In each experiment there was the same number of control and experimental fish, and like numbers of fish of approximately the same size. The controls received the same treatment as the experimentals save that hormone was not administered to the controls. For each experiment the environment was identical for the control and experimental groups. These groups were segregated in salt-water tanks. For Experiments 1, 2, 4 the water temperatures were in the main near 13°–14° C.; for Experiment 3 the water temperature was 17°–19° C. Testes were trimmed and weighed after fixation in Bouin's fluid. The precise treatments for each experiment will be found in the *Results*.

[1] Testosterone propionate (Perandren), and testosterone were kindly supplied by the Ciba Pharmaceutical Products, Inc.

Results

The periods covered by these experiments were from the full breeding state (June and July) to complete testicular involution and the preliminary spermatogonial multiplications for a new cycle (August–November). In *Fundulus* there is no abrupt transition from the breeding to the involuted condition in the testis. The gonads do not suddenly cease to form sperm; even when the testes have involuted to almost minimal size, a few sperm continue to be formed. Instead, there is a gradual decline from July to September in the number of sperm formed. The male ducts which are almost wholly intra-testicular in *Fundulus*, also slowly involute from a condition where the ducts are long and distended with sperm to one where they have degenerated into a mass of stromal tissue in which individual ducts can no longer be recognized.

The yellow coloration of the body, characteristic of the breeding male, likewise slowly disappears. Not until late August do the males completely lose this coloration, which is most obvious on those parts of the body where there is the least melanin pigmentation (ventral surface and fins). In the fairly cold water used in Experiments 1, 2, 4, these regressive changes go more slowly than they do in the warmer water of the natural habitat.

Exp. 1. Between June 27 and August 2, twenty-eight adult males, hypophysectomized on June 23–24, each received intraperitoneally, ap-

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PLATE I

Explanation of Figures

Fig. 1. Cross-section of a testis from a breeding male at the time when hypophysectomies were performed. The peripheral zone of spermatogenetic activity (s.z.) is broad. The more central duct system (d.) is well developed. Black areas are sperm.

Fig. 2. Cross-section of a testis from a fish kept in a cold-water laboratory tank 6/23–8/8. The spermatogenetic zone is still well developed, but the duct system shows some involution. The testis is smaller than that of a breeding male. Sections from testes from fish injected with 8 mg. testosterone show the same condition as shown in this figure.

Fig. 3. Cross-section of a testis from a control hypophysectomized fish killed 8/1. The spermatogenetic zone (s.z.) is almost devoid of spermatids. The testis is very much smaller than that shown in Figure 2.

Fig. 4. Cross-section of a testis of a control hypophysectomized fish, killed 8/8. Involution is more advanced than that shown in Fig. 3.

Fig. 5. Cross-section of a testis from a hypophysectomized fish, 8/1, which received intraperitoneally 7 mg. of testosterone propionate in oil. Spermatogenesis is more in evidence than in the controls (cf. Fig. 3). The ducts are also better maintained.

Fig. 6. Cross-section of a testis from a hypophysectomized fish, 8/8, which was injected intraperitoneally with 8 mg. of testosterone propionate. The spermatogenetic zone (s.z.) is less active than on 8/1 (Fig. 5), but the duct system (d.) is still well developed.

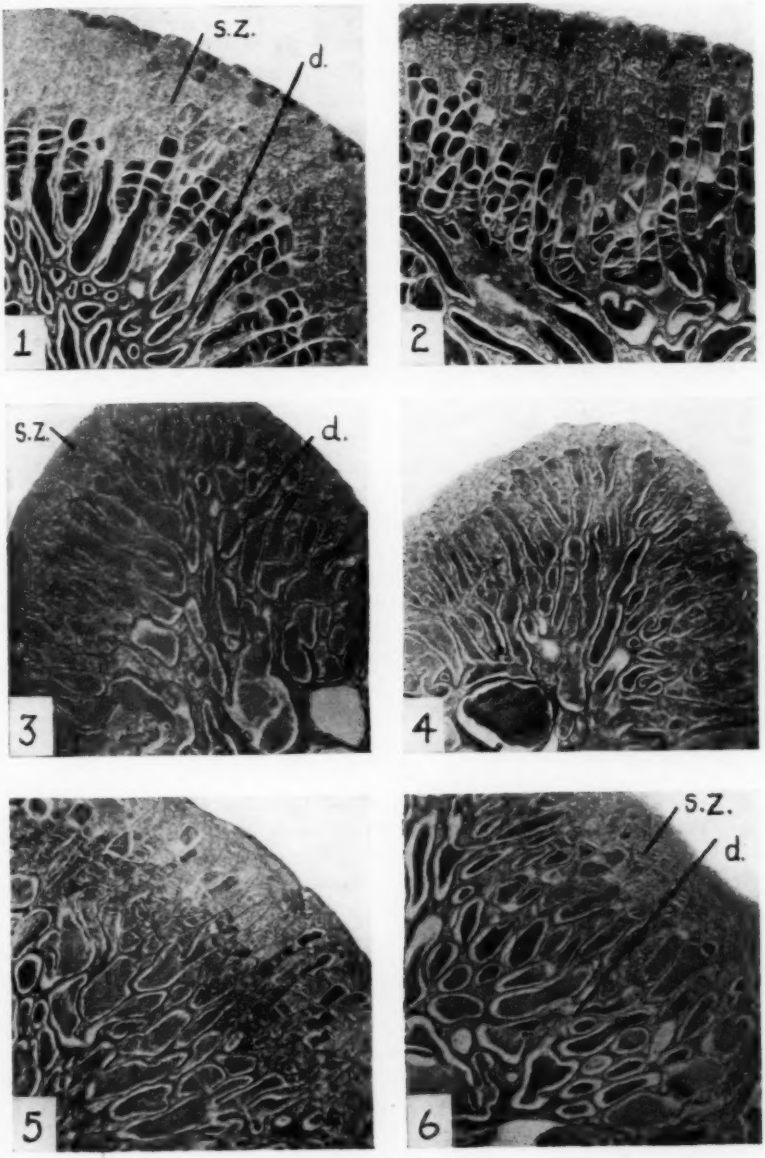

PLATE I

proximately 8 mg. of testosterone propionate dissolved in sesame oil, in equally spaced doses of 1 mg. Fish were sacrificed on August 1 and 8.

At the time of hypophysectomy, the testes were actively forming sperm (Fig. 1); the peripheral zone of spermatogenetic transformations (s.z.) is deep. The more centrally located ducts (d.) are distended and elongate. A section of the testis of a normal laboratory fish at the time of the end of the experiment (Aug. 8) is shown in Figure 2. The only difference between this testis and one from the breeding season (Fig. 1) is a slackening of spermatogenetic activity and a very slight involution of the duct system. The testicular states present in the hypophysectomized controls on August 1 and 8 are seen in Figures 3 and 4. Here spermiogenesis has almost ceased; only scattered cysts of spermatids can be found. The duct system has been markedly reduced. The testes of the hypophysectomized controls were only half as heavy as those of normal controls.

The hypophysectomized fish which were injected with 8 mg. of testosterone propionate over a 42 day period, had testes which weighed[2] about one-fifth more than those of hypophysectomized controls. Cross sections of the testes of hormonally treated fish are shown in Figures 5 and 6. It is obvious that testosterone propionate has not maintained the testes as well as they were maintained in normal fish (cf. Fig. 2). The involution is not as marked however, as in the hypophysectomized controls (cf. Figs. 3 and 4). Stages of spermatogenesis are more in evidence and the duct system is better preserved. While these differences between hormonally treated and control fish are constant, the testes of the treated fish are also near the end of their spermatogenetic activity. The spermatogenetic-stimulating effects of testosterone propionate with the methods used, are slight. The regressive changes due to hypophysectomy far outweigh any gametogenetic effect of the hormone.

The hormonally treated hypophysectomized fish maintained the full yellow breeding coloration, while most of this coloration was lost in the hypophysectomized controls. Since the nuptial mark (Parker and Brower, 1935) is lost more slowly than is the yellow coloration, a positive statement on this character cannot be made. The contact organs (Newman, 1907) were not studied.

Exp. 2. Between June 27 and August 2, twenty normal males each received intraperitoneally, approximately 8 mg. of testosterone dissolved in oil, in spaced doses of 1 mg. Animals were sacrificed August 1 and 8.

No differences in testicular states were found after a 42 day period between the control and hormonally treated fish. Figure 2 illustrates

[2] The comparisons of testicular weights must be considered only as rough comparisons.

the condition of these fishes' testes. The hormonally treated fish retained the yellow body coloration better than the controls but less well than it was retained by the hypophysectomized males treated with testosterone propionate.

Exp. 3. Between October 9 and 29, six normal males were injected intraperitoneally with approximately 6 mg. of testosterone propionate in oil in spaced doses of 1.5 mg. Animals were killed November 3.

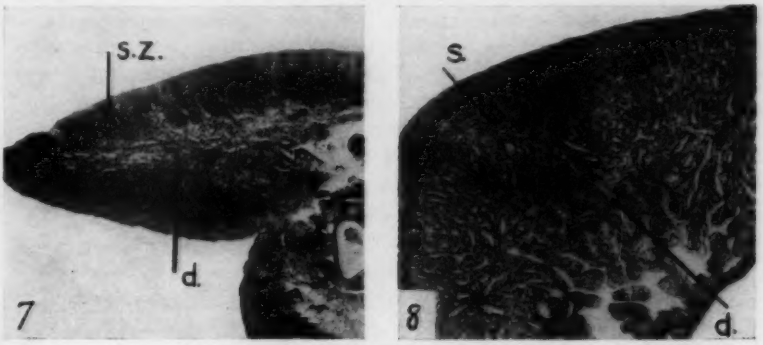

PLATE II

EXPLANATION OF FIGURES

FIG. 7. Cross-section from a normal control male, 11/3, after 25 days in a laboratory tank. The ducts (d.) are represented by the central stromal core, about which is the spermatogonial zone (s.z.) in the early stages of spermatogonial multiplication.

FIG. 8. Cross-section of a testis from a normal fish, 11/3, injected intraperitoneally with 6 mg. of testosterone propionate. The duct system (d.) has hypertrophied and individual ducts are visible. The spermatogenetic zone shows spermatids (s.). Essentially the same testicular state was present in fish receiving 3 mg. of testosterone propionate intramuscularly.

On October 9 and 25, six normal males each had approximately 3 mg. (2 doses of 1.5 mg.) of crystalline testosterone propionate implanted into the dorsal musculature. To effect this implantation, crystals of the hormone were tamped into the tip of a hollow glass needle. A wire plunger, snugly fitting the bore of the needle, forced the hormone into the muscles. One scale was removed from the fish. At the site of removal the needle was inserted. The scale was replaced to cover the wound. Animals were sacrificed on November 3.

A section of a normal control's testis is shown in Figure 7. Such a fish was kept 25 days in a laboratory tank. The testicular involution of the previously active gonad has been accomplished, and the prolifera-

tion of spermatogonia for a new cycle has started. Spermatids are as yet rare. The testes of the fish treated with 6 mg. of intraperitoneally injected hormone were about four times as heavy as those of the controls. The testes of the fish in which 3 mg. of testosterone propionate were implanted intramuscularly were about twice as heavy as the controls.

The histological picture was essentially the same for both groups of treated fish, with the testes from the fish injected intraperitoneally being the more advanced. Figure 8 shows a section from a testis from a fish which received intraperitoneal injections of the hormone. The most conspicuous effect of the hormone was the re-elaboration of the duct system of the gonad. Instead of being represented as in the controls by a mass of stroma (Fig. 7, d.), the individual ducts have become individually discernible. The walls of the ducts are thickened and ciliated. The duct tissue now forms the bulk of the testis and is responsible for most of the increased weight of the gonad.

The effect of the hormone on spermatogenesis was again present, but again not marked. In the treated fish there was a slight acceleration of spermatogenesis. This acceleration was not enough to present widely differing pictures between the germ cells of the controls and the hormonally treated fish. While spermatids were much more numerous in the treated fish than in the controls, numerous spermatozoa were not found. It was shown (Burger, 1941) that pituitary material could induce numerous spermatozoa in colder water and in a shorter length of time than the 25 days of this experiment.

The effect of the hormone on the yellow coloration of the body was clear. Within ten days after the first injection, yellow had developed on the body and the fins. At the end of the experiment the hormonally treated fish were brilliantly yellow. In the same period, the controls remained devoid of this color, save for one fish which showed the faintest tint. It must be remembered that the controls were slowly undergoing sexual stimulation from the warm water of the experiment.

Exp. 4. Beginning August 16, ten normal males each received intraperitoneally, approximately 9 mg. of testosterone propionate in oil in 3 mg. doses spaced at intervals of seven days. Fish were sacrificed on September 3.

On August 15, six normal males had approximately 1.5 mg. of testosterone propionate implanted into the dorsal musculature. These animals were killed on September 3.

The results of these experiments were essentially similar to those of Experiment 3. After 18 days of intraperitoneal treatment with hormone the testes were half again as heavy as those of the controls. The fish treated with intramuscular hormone had testes two-fifths heavier

than those of the controls. The hormonally treated fish were more yellow than the controls.

Discussion

In these experiments effects of testosterone propionate were observed upon the yellow coloration of the body, the intra-testicular duct system, and the germ cells. This hormone maintained the full yellow coloration of the breeding male for 42 days after hypophysectomy. In the hypophysectomized controls this coloration was largely lost. In normal sexually inactive males, testosterone propionate, injected either intraperitoneally or intramuscularly, caused a re-appearance of this yellow coloration. Controls did not become similarly colored. In normal breeding males, testosterone, milligram for milligram, was less effective than was testosterone propionate in hypophysectomized males. The results indicate that the suffusion of the body with yellow during the breeding period is normally caused in the adult male *Fundulus* by an androgenic substance. Female *Fundulus* do not show this coloration.

Within the testis the most conspicuous effect of testosterone propionate was upon the duct system. In normal sexually inactive fish the hormone produced a marked hyperplasia of the ducts. In hypophysectomized males, the hormone, with the methods used, only partially maintained the duct system. The duct system was, however, better maintained than in the controls. Testosterone with the methods used had no discernible effect on the duct system.

The effects of testosterone propionate on the germ cells are not unequivocal. In the mammal it has been shown that large doses of androgens maintain the normal condition of the testis if the injections are given before degenerative damage has occurred (Walsh, Cuyler and McCullagh, 1934; Nelson and Gallagher, 1936; Hamilton, 1936; Cutuly, McCullagh and Cutuly, 1937). In the normal adult *Lebistes*, Eversole (1939, 1941) reports somewhat contradictory results. In his 1939 paper testosterone propionate was found to have no effects in experiments of short duration and to be inhibitory to spermatogenesis in experiments of three months duration. In his 1941 paper the hormone is said to have the effects of pregneninolene, which latter substance hastens germ cell maturations without replacements by new cells and causes a hyperplasia of the testicular stroma. Eversole considers the male hormone of *Lebistes* to be more similar to pregneninolene than to testosterone propionate. In the lizard, *Sceloporus*, Forbes (1941) finds that implanted pellets of testosterone propionate stimulate spermatogenesis and produce a hypertrophy of the epididymis and vas deferens. He criticizes the

negative results of Gorbman (1939) as being the result of too short an experiment.

Our results indicate that testosterone propionate has, within the methods used, only a very slight stimulating effect on the male germ cells of *Fundulus*. This effect did not suppress the involution changes that follow hypophysectomy, although the hormone did very slightly retard this involution. In normal sexually inactive fish, this hormone did not markedly accelerate a new spermatogenesis. We have shown (Burger, 1941) that within the time limits of these experiments, pituitary material will produce copious sperm in normal and hypophysectomized *Fundulus*. It is interesting to note that the response to implanted whole pituitaries is primarily one of germ cell activation, and not one of stimulation of the ducts. With testosterone propionate, the effect seems to be the reverse, i.e., marked activation of the duct system and weak activation of the germ cells. The effect noted on the germ cells may not be a direct effect at all. It may be that by stimulating the duct system, which is intra-testicular, the *milieu* of the testis is more favorable for spermatogenesis. It must be remembered that in these experiments the germ cells of the treated fish were not in stages which differed widely from the controls.

It next must be asked whether or not the methods and the dosages employed were adequate for securing pertinent results. In comparison with other data from other workers the use of at least 1 mg. of testosterone propionate per 7.5–9.5 gm. fish per week seems to be a fairly high dosage. The use of intraperitoneal injections of the hormone in an oil vehicle may be questioned on the grounds that it does not provide for a satisfactory absorption of the hormone. Deansley and Parkes (1937) report that intraperitoneal testosterone has little effect in the mammal. Rubinstein and Kurland (1941) do not agree with this conclusion, but Greene and Burrill (1941) cast doubt on the validity of the comparisons made by Rubinstein and Kurland. Experiment 3 clearly shows that in *Fundulus* 6 mg. of intraperitoneal testosterone is somewhat more effective than 3 mg. of intramuscular hormone. The hormone in both methods of administration affected in like fashion the testis and the body coloration. Thus, it can be stated that effective amounts of testosterone propionate can be absorbed after intraperitoneal and intramuscular administration. It is clear from Experiment 2, that the amount of testosterone used was inadequate to produce testicular stimulation.

The intraperitoneal method of administration of the hormone is for small fish more convenient than are intramuscular and subcutaneous methods. In these latter two methods the repeated injection of large

doses is not practical, due to the wounds inflicted and the delicateness of the dermal epithelium. Since *Fundulus* is a very wasteful feeder with non-living food, the oral administration of the hormone would be wasteful of the hormone, and would make a reasonably accurate determination of the dosage difficult.

Summary

Adult male *Fundulus* hypophysectomized during the breeding season were injected intraperitoneally with 8 mg. of testosterone propionate during a 42 day period. The hormone maintained the yellow body coloration characteristic of a breeding male. The hormone did not maintain the testis in the breeding condition. The testicular ducts were maintained better than they were in the controls. There was slightly more spermatogenetic activity than in the controls. Like amounts of testosterone injected intraperitoneally into normal fish over a similar period had no effect on the testis and only partially maintained the yellow coloration of the body.

Normal adult male fish injected intraperitoneally or intramuscularly with testosterone propionate (see text for dosage) during testicular involution and during the early stages of spermatogonial multiplications showed a marked activation of the testicular ducts and a slight stimulation of spermatogenesis. The yellow coloration of the body developed brilliantly in the treated fish. The above experiment was performed once for an 18 day period, and once for a 25 day period.

It is concluded that the yellow coloration of the body, characteristic of the breeding male, is stimulated by the male hormone, and the elaboration of the testicular duct system is influenced by this hormone. While a slight stimulation of spermatogenesis was observed, the degree of stimulation does not warrant the conclusion that in the intact animal the male hormone has any important spermatokinetic role.

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FACTORS INFLUENCING REGENERATION AND POLARITY DETERMINATION IN TUBULARIA CROCEA

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The regeneration of hydroids has been studied extensively, and the subject has recently been reviewed by Barth (1940*b*). Of the environmental factors which effect regeneration in the hydroids, the role of oxygen has been studied in the most detail (Miller, 1937; Barth, 1938*a*; Zwilling, 1939; Rose and Rose, 1941). Several evidences have been presented, however, which indicate that the accumulation of waste products may have an inhibitory effect on regeneration. When the cut ends of *Tubularia* stem segments are covered with glass capillaries, regeneration is inhibited at the covered ends (Barth, 1938*a*). Rose and Rose (1941) showed that accumulated waste products may inhibit regeneration at a cut surface of *Tubularia,* even when a plentiful supply of oxygen is available at the cut surface. Komori (1933) reported that lowered pH may inhibit regeneration. Miller (1939) reversed the normal polarity of *Tubularia* stems by treating the distal ends with a 90 per cent O_2: 10 per cent CO_2 mixture, while the proximal ends were exposed to oxygen.

Attempts to analyze the role of environmental factors in regeneration and the origin of polarity during regeneration are complicated by the heterogeneous nature of the *Tubularia* stem. In the *Tubularia* stem there is a gradient in the rate of regeneration (Barth, 1938*b*), as well as a gradient of oxygen consumption (Barth, 1940*a*). Further complication is introduced by the dominance which distal levels of the stem exert over proximal levels (Barth, 1938*b*). In order to obtain a clear analysis of the role of the environment in regeneration, it is advantageous to work with a homogeneous system. Such a system is provided by the *Tubularia* coenosarc fragments described by Goldin and Barth (1941). When the coenosarc is removed from the perisarc, a series of reorganizational changes occurs involving both morphological and physiological dedifferentiation. A spherical mass of coenosarc cells is formed, in which there is a complete loss of the original polarity of the stem. The polarity relationships which are developed during the regeneration of the coenosarc fragment are therefore new, and have no relation to the original polarity present in the intact stem.

The experiments, using both coenosarc fragments and stem segments, were designed in order to study the effects of oxygen and accumulated waste products in regeneration. They may be described under the following headings: (1) inhibition of regeneration inside of glass caps; (2) determination of polarity by the differential exposure of coenosarc; (3) the extent of acid metabolite accumulation; and (4) the effect of increased hydrogen ion concentration on regeneration. The author wishes to thank Professor L. G. Barth, under whose supervision this work was carried out, and Professor H. B. Steinbach for his helpful suggestions in the preparation of the manuscript.

Methods Employed in the Capillary Experiments

The method of treatment of the coenosarc fragments was essentially the same as that described by Goldin and Barth (1941). Segments of stem ten millimeters long were cut from the region of *Tubularia* stems extending from five to fifteen millimeters proximal to the hydranth. The coenosarc tissues were expressed from the perisarc and were kept on agar (2 per cent agar in sea water) in running filtered sea water, until ready to be used in the experiments. When stem segments were used, they were taken from the same region of the stem (5–15 mm. proximal to the hydranth), but the perisarc was not removed. The capillaries were drawn from clean glass tubing (7 mm. inside diameter) to a diameter of approximately one millimeter. The capillaries were cut into 25 mm. lengths, and when ready for use, were filled with filtered sea water. A capillary was chosen for each coenosarc fragment so that the fragment would fit tightly enough not to be dislodged during the course of the experiment. In order to manipulate the coenosarc fragments without injury to them hair loops were employed. After the coenosarc fragments were inserted into the capillaries, the latter were submerged in running filtered sea water for the duration of the experiment. The capillaries were all kept one half inch below the surface of the water in order to insure uniformity of conditions. The temperature of the sea water during the experiments was $18 \pm 2°$ C. The operations and observations were made with the aid of a binocular microscope.

Inhibition of Regeneration Inside of Glass Caps

Barth (1938*a*) found that regeneration was inhibited when the cut end of a *Tubularia* stem was covered with a glass capillary. This experiment was performed in order to determine whether inhibition could likewise be effected using coenosarc fragments. Dedifferentiated coenosarc

fragments (twenty-four hours after removal from the perisarc) were inserted in capillaries, midway between the two open ends. Stem segments ten millimeters long were placed in the center of similar capillaries. Other coenosarc fragments and stem segments were kept in running filtered sea water as controls.

Regeneration was inhibited in all cases when the coenosarc fragments and stem segments were covered with capillary tubing. Thirty-one coenosarc fragments failed to regenerate one hundred and fifty hours after being placed in the capillaries (174 hours after removal of the coenosarc from the perisarc). Twenty-three out of twenty-five coenosarc fragment controls regenerated ninety hours after removal of the coenosarc from the perisarc. Ten stem segments placed in capillaries showed no evidences of regeneration after one hundred and forty-two hours, while all of the twenty control stem segments regenerated after seventy hours. When coenosarc fragments and stem segments were removed from the capillaries, many of them regenerated, indicating that the inhibition is reversible.

Determination of Polarity by the Differential Exposure of Coenosarc

Since the covering of a coenosarc fragment by capillary glass tubing is sufficient to cause inhibition, a differential exposure could be accomplished by placing a coenosarc fragment inside the tip of a capillary so that half of the surface is covered by the capillary while the other half is exposed to the sea water in the dish. The coenosarc frament were placed in the capillaries at time intervals ranging from twenty-four to forty-eight hours after removal from the perisarc. Coenosarc fragments and stem segments were kept in running filtered sea water as controls.

The results of these experiments are summarized in Table I. Seventy-nine coenosarc fragments placed in the tips of the capillaries regenerated new hydranths at the exposed surface of the fragment, while none regenerated at the capped surface. Thus, all regenerants were unipolar, and polarized in the direction of the open end of the capillary. The control coenosarc fragments, on the other hand, gave rise to unipolar, bipolar, bipolar-unipolar, and multipolar regenerants, as described by Goldin and Barth (1941). Since a coenosarc fragment, at twenty-four hours after removal from the perisarc has lost its original polarity relationships (Goldin and Barth, 1941), the polarity of the regenerant is new, and is developed in response to the imposed environmental differential. It may be noted that, although at 48 hours after removal from the perisarc, coenosarc fragments have begun to redifferentiate (Goldin and

Barth, 1941), nevertheless, the differential exposure still determines the polarity of these regenerants. It is indicated, therefore, that the redifferentiation has not yet produced an irreversible determination of polarity.

The factors involved in the differential exposure appear to be oxygen and metabolic waste products. The diffusion rate of oxygen to the coenosarc tissue and of waste products away from the tissue is presumably lower inside the capillary than at the open end. The extent of this effect is indicated by the experiment of Barth (1938*a*), where inhibition was obtained even when the cut end of the *Tubularia* stem was flush with the end of the capillary. On this basis the coenosarc tissue is subjected to two gradients. There is a gradient of oxygen tension, with the high end at the exposed surface of the tissue, and a gradient of concentration of metabolic wastes, with the high end at the covered surface of the tissue.

TABLE I

The polarity developed by coenosarc fragments placed in the tips of glass capillaries T_1 and T_2 = time in hours after perisarc removal. T_1 = time at which the fragments were placed in the capillaries, T_2 = time at which the observations were made.

Description			Polarity of the regenerants			
Exp.	No. of fragments	T_1	T_2	Regeneration at exposed surface	No regeneration	Dead
1	43	24	96	28	7	8
2	20	39	135	7	2	11
3	18	43	88	4	8	6
4	16	48	163	10	3	3
5	48	48	192	30	12	6
Total	145			79	32	34

Either one, or a combination of both of these gradients might determine the new polarity relationships which develop during regeneration. If an oxygen gradient is the sole factor acting in the determination of the new polarity, it should be possible to obtain regeneration at any point on the coenosarc tissue by the application of a high oxygen tension at any localized point on the tissue, irrespective of the accumulation of waste products. Three experiments were designed in order to test the efficacy of oxygen in the presence of accumulated metabolic wastes.

In the first experiment dedifferentiated coenosarc fragments (26 hours after removal from the perisarc) were placed in the tips of capillaries so that half of the tissue surface was exposed to the sea water in the dish, while the other half was covered with the glass capillary. An oxygen bubble was then introduced into the capillary by means of a micropipette,

at a distance of from two to four millimeters from the coenosarc fragment. The size of the oxygen bubble was such as almost to fill the capillary.

The observations were made 96 hours after the coenosarc fragments were placed in the tips of the capillaries. Out of a total of 37 fragments treated in this way, 14 regenerated at the free end of the capillary, two regenerated in the direction of the oxygen bubble, 11 failed to regenerate, and ten died. Control coenosarc fragments, placed in the tips of the capillaries as in the previous experiment, with no oxygen bubble introduced into the capillary, all regenerated towards the free end of the capillary.

In the second experiment, dedifferentiated coenosarc fragments were placed in the center of glass capillaries containing sea water. An oxygen bubble was then introduced into one end of the capillary, and a nitrogen bubble into the other end of the capillary. The oxygen and nitrogen bubbles were of approximately the same length (8 mm.) and were placed about 2 mm. from the coenosarc fragment. Twenty coenosarc fragments (30 hours after removal from the perisarc) were treated in this way. After 120 hours none of the coenosarc fragments had regenerated. Seven out of ten untreated control coenosarc fragments, free in sea water, regenerated during the same period. It may be noted that the gas bubbles introduced into capillaries showed no appreciable loss in volume during the experiment.

In the final experiment, the coenosarc was removed from the perisarc of the distal half of ten millimeter stem segments, the empty perisarc being left intact. An oxygen bubble was then introduced into the empty half of the perisarc, and the stems were ligated at both cut ends. Untreated stem segments, as well as stem segments from which the coenosarc of the distal half was removed, but in which the ends were ligated without the introduction of an oxygen bubble, were kept as controls. All of the stems were kept in running filtered sea water at $19 \pm 1°$ C.

A total of six out of 60 experimentals regenerated hydranths after 96 hours, in the direction of the oxygen bubble. The oxygen bubbles themselves were reduced, but in no case completely dissipated during the experiment. None of the ligatured controls regenerated during the same period; whereas 29 out of 40 cut, but unligatured, stems regenerated. The experimentals which did regenerate apparently did so because of the increased availability of oxygen. Failure of ninety per cent of the stems to regenerate, despite the presence of the oxygen bubble, was probably due to the inhibition caused by the accumulation of waste products.

The Extent of Acid Metabolite Accumulation

This experiment was designed in order to determine the extent to which acid metabolites accumulate when regeneration is inhibited by capping. This could be accomplished by measuring the change in the pH of the sea water inside of the glass tubing. Clean seven millimeter glass tubing was cut into 24 millimeter lengths, and submerged in filtered sea water. Both stem segments and coenosarc fragments were used, one segment or one fragment being inserted into each tube. The ends of the tubing were then plugged with absorbent cotton in order to prevent the stem segments and coenosarc fragments from falling out. Measurements of pH were made with a Beckman pH meter, the water contained in five glass tubes being pooled for each reading. Coenosarc fragments and stem segments were kept in standing sea water as controls.

Twenty-five coenosarc fragments, and twenty-two stem segments were placed in the glass tubes. As in the capillaries, there was complete inhibition of regeneration. The average pH of the sea water in the glass tubes containing coenosarc fragments dropped from 7.96 to 7.11 after 96 hours. With stem segments, the average pH was lowered from 7.96 to 6.90 during the same time interval. Twenty-one out of 35 control fragments regenerated, the pH of the sea water changing from 7.96 to 7.86, while 15 out of 20 control stem segments regenerated with a pH change from 7.96 to 7.90. Thus, associated with the inhibition, there is a lowering of the pH of the sea water.

The Effect of Increased Hydrogen Ion Concentration on Regeneration

The results of the previous experiments indicated that the accumulation of acid metabolites has an inhibitory effect on regeneration. If the inhibitory action of acid metabolites is due to the increased hydrogen ion concentration of the sea water, it should be possible to obtain inhibition by artificial acidification of the sea water. For an accurate analysis it is necessary to maintain the oxygen tension of the sea water samples at a constant level, while the acidity is varied. The general procedure for adjusting the sea water samples to the desired pH values was essentially the same as that used by Smith and Clowes (1924), and Whitaker (1937), the pH being regulated with acid, base, and buffer. An attempt was made to get a range of pH values from that of normal sea water (pH 8–8.2) down to pH 3. After the addition of acid, the sea water was aerated vigorously in order to re-equilibrate with the atmosphere. This was necessary, since the addition of acid liberates free carbon dioxide.

The pH measurements were made, as before, with a Beckman pH meter, and the dissolved oxygen was determined using the Winkler technique. Two series of experiments were performed.

In the first series of experiments the sea water was acidified to pH 3 by the addition of concentrated HCl. Air was then bubbled vigorously through the sea water for six hours, after which time successive measurements gave constant pH readings. Measured quantities of sea water (100 cc.) were then adjusted to a series of pH values by the addition of the following reagents: 0.2M NaOH; 0.6M $NaHCO_3$; 0.2M NaOH—0.6M $NaHCO_3$; and McIlvaine's buffer (Citric acid 0.1M— secondary sodium phosphate 0.2M). The sea water made up to the different pH values was poured into two-inch finger bowls placed in a constant temperature bath at 19 ± 1° C. Stem segments taken from the region 5–15 mm. proximal to the hydranth were placed in each finger bowl and samples of sea water removed for determination of pH and dissolved oxygen. The stems were observed for regeneration when, by gross inspection, most of the control stems in normal sea water had formed primordia. The pH and the oxygen tension were determined again at the close of the experiment.

In a second series of experiments the sea water was acidified to the desired pH by the addition of concentrated hydrochloric acid. Air was then bubbled through the acidified sea water samples until constant pH readings were obtained. Stem segments were introduced into these sea water samples as in the previous experiment.

The results are summarized in Table II. As the hydrogen ion concentration of the sea water is increased, fewer stems regenerate during a given period. After 55 hours, 19 out of 20 stem segments regenerated at pH 8.08–8.04 (normal sea water), while only eight out of 20 stem segments regenerated at pH 7.05–7.04 (Table II, Exp. 3). The inhibitory effect was more pronounced as the pH was lowered, so that at approximately pH 6 no regeneration occurred. After 60 hours, four out of ten stems regenerated at pH 6.02–6.29, while at pH 5.93–6.08 there was complete inhibition (Table II, Exp. 1). The stems at the latter pH were kept in the experimental medium for a longer period (95 hours), at which time they still showed no signs of regeneration. As low as pH 6, the inhibitory effect is reversible, inhibited stems regenerating when returned to normal sea water. Below pH 6 the coenosarc tissue of many of the experimentals appeared to undergo gradual cytolysis and no recovery occurred. It may be noted that the oxygen concentration of the sea water samples was approximately the same for each experiment, and

that the concentration is in the range usually found in normal sea water. This rules out the possibility that inhibition was due to lack of oxygen.

Discussion

Coenosarc fragments fail to regenerate when they are inserted into capillary glass tubing. A similar effect was demonstrated by Barth

Table II

The Effect of Increased Hydrogen Ion Concentration on the Regeneration of Tubularia Stem Segments

Medium	Initial pH	Final pH	Initial O_2 cc./l.	Final O_2 cc./l.	Number of regenerants
Experiment 1. Observed at 60 hours 10 stems at each hydrogen ion concentration					
Normal sea water	7.93	7.88	4.4	4.2	8
Acid S.W. + $NaHCO_3$	7.89	7.94	4.0	4.1	7
Acid S.W. + NaOH–$NaHCO_3$	7.87	7.42	4.4	4.1	7
Acid S.W. + NaOH	6.02	6.29	4.0	4.2	4
Acid S.W. + McIlvaine's	5.93	6.08	4.4	4.1	0
Acid S.W. + McIlvaine's	5.44	5.90	4.2	4.1	0
Experiment 2. Observed at 72 hours 10 stems at each hydrogen ion concentration					
Normal sea water	7.89	7.77	4.6	5.0	9
S.W. + HCl	6.77	7.23	4.6	4.9	7
S.W. + HCl	6.71	7.08	4.7	4.9	6
S.W. + HCl	6.57	7.19	4.6	4.8	6
S.W. + HCl	6.39	6.84	4.6	4.8	5
S.W. + HCl	4.88	5.70	4.8	4.5	0
S.W. + HCl	3.28	3.26	4.8	5.0	0
Experiment 3. Observed at 55 hours. 20 stems at each hydrogen ion concentration					
Normal sea water	8.08	8.04	4.6	4.6	19
S.W. + HCl	8.00	7.93	4.8	4.8	16
S.W. + HCl	7.91	7.74	4.7	4.7	14
S.W. + HCl	7.82	7.76	4.7	4.7	15
S.W. + HCl	7.62	7.56	4.7	4.7	11
S.W. + HCl	7.05	7.04	4.7	4.7	8

(1938*a*) using cut stems with perisarc, where covering of the cut end with glass tubing was sufficient to cause inhibition. The capillary walls decrease the diffusion rate and this might cause the oxygen tension of the sea water to fall below the level required for regeneration. In addition,

acid metabolites would accumulate in the sea water surrounding the tissues. Either one of these factors, or their combination, might cause the inhibition.

When the coenosarc fragments are placed in the tip of a capillary so that half of the tissue surface is covered by the capillary, while the other half is exposed to the sea water in the dish, regeneration always occurs at the exposed surface. Thus, the new polarity relationships developed by the homogeneous mass of tissue may be strictly determined as the result of a differential exposure to the environment. The differential exposure probably consists of two gradients, a gradient of oxygen tension, with the high end at the open end of the capillary, and a gradient of acid metabolite concentration, with the high end inside the capillary.

That oxygen plays a necessary role in regeneration has been clearly elucidated by other investigators. There is a threshold of oxygen tension below which regeneration will not occur, while above this threshold, the rate of regeneration is a function of the oxygen tension (Barth, 1938*a*). Miller (1937) obtained a reversal of polarity of *Tubularia* stem segments when the proximal cut end was bathed with oxygen and the distal end was bathed with nitrogen. Here, since the active bubbling of oxygen and nitrogen would remove waste products at the cut ends, the effect of oxygen was probably not vitiated by the inhibitory action of accumulated acid metabolites. The effectiveness of oxygen in regeneration is, however, limited by the inhibitory action of accumulated acid metabolites. This view is supported by the following evidences.

Most of the coenosarc fragments placed in the tips of capillaries regenerate in the direction of the exposed surface, despite the presence of a large oxygen bubble inside of the capillary. It may be assumed that the oxygen supply inside of the capillary was at least equal to that provided by normal sea water, so that regeneration should occur inside of the capillary. Since waste products presumably accumulate inside of the capillary, but are removed at the free end, it is indicated that the polarity of the regenerants was determined by the differential exposure to metabolic wastes. Thus, when there are no appreciable differences of oxygen tension at localized portions of the coenosarc fragments, a differential of the concentration of waste products may determine the polarity of the regenerants.

Coenosarc fragments inserted in glass capillaries do not regenerate when an oxygen bubble is placed on one side of the fragment and a nitrogen bubble on the other side. Failure to regenerate is probably due to the accumulation of acid metabolites, for again, sufficient oxygen was apparently available for regeneration to occur under normal conditions.

This interpretation is supported by the experiments of Rose and Rose (1941) in which it was demonstrated that regeneration is inhibited despite the presence of an oxygen bubble, when a cut end of a *Tubularia* stem is inserted into a tight-fitting cap. If the cap is loose-fitting, regeneration may occur.

Further evidence is offered by the experiments in which oxygen bubbles were introduced into stem segments. The coenosarc tissue was removed from the distal half of *Tubularia* stem segments. An oxygen bubble was then introduced into the empty half of the perisarc, and the cut ends were ligated. Ninety per cent of these stem segments failed to regenerate in running sea water, despite the presence of the large oxygen supply. This inhibition was probably caused by the accumulation of waste products. The stems which did regenerate, formed the hydranth at the distal end of the segment. Rose and Rose (1941) injected oxygen gas into the coelenteron of *Tubularia* stem segments and then ligated the ends. Twenty-one per cent of the stems regenerated when they were kept in running sea water, whereas none of the stems regenerated when they were kept in still water. Their results are understandable if waste products are removed more rapidly in an actively circulating medium.

The extent to which acid metabolites may accumulate as a result of capping is demonstrated by the change in pH of the sea water contained in glass tubing to which coenosarc fragments and stem segments had been added. In addition to inhibition of regeneration, there is a lowering of the pH of the sea water. With coenosarc fragments, the average pH of the sea water dropped from 7.96 to 7.11 after 96 hours, while with stem segments the average pH dropped from 7.96 to 6.90.

The acid metabolites probably cause inhibition by increasing the hydrogen ion concentration of the medium. The inhibitory action of increased hydrogen ion is demonstrated by the results of the experiments in which the pH of the sea water was regulated with acid, base, and buffer. The ability of *Tubularia* stem segments to regenerate is a function of the hydrogen ion concentration of the medium. At the oxygen tension of normal sea water, there is a range of pH in which regeneration is retarded, and a critical pH below which regeneration will not occur. The critical pH is probably related to the oxygen tension. Inhibition of stem regeneration in glass tubing occurred at a higher pH (pH 6.90) as compared with stems kept in open dishes (pH 5.93–6.08). This difference may be accounted for, if, in the glass tubing, a lowering of the oxygen tension of the sea water occurred concomitantly with the accumulation of acid metabolites. This would mean that the oxygen tension of the sea water required for regeneration is a function of the hydrogen

ion concentration. If this is true, it is suggested that the interrelationship of these factors in influencing *Tubularia* regeneration may be worked out in detail by studying regeneration in sea water at varying oxygen and hydrogen ion concentrations.

Summary

Environmental factors effecting regeneration and the origin of polarity in regeneration were investigated. The experiments were performed using coenosarc fragments and stem segments of *Tubularia crocea*.

Regeneration was inhibited when coenosarc fragments and stem segments were inserted into glass tubing. Concomitant with the inhibition, there was a measurable drop in the pH of the sea water contained in the tubing.

Coenosarc fragments inserted into the tips of capillaries regenerated at the exposed surface of the fragments. The same polarization resulted in most cases, even when large oxygen bubbles were introduced into the capillaries.

Coenosarc fragments placed in capillaries, failed to regenerate, when an oxygen bubble was placed on one side of the fragments and a nitrogen bubble on the other side of the fragments.

When oxygen bubbles were introduced into the emptied distal portions of *Tubularia* stem segments, and the cut ends of the stems were ligated, only ten per cent of the stems regenerated.

All of these results support the view that the effectiveness of oxygen in regeneration may be limited by the inhibitory action of accumulated acid metabolites.

Acid metabolites probably cause inhibition by increasing the hydrogen ion concentration of the medium, for at the oxygen tension of normal sea water, there is a range of pH in which regeneration is retarded, and a critical pH below which regeneration will not occur.

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FURTHER STUDIES IN THE PHARMACOLOGY OF THE HEART OF CANCER MAGISTER DANA

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In earlier work (Davenport, 1941), it was found that there is a marked similarity between the effects of nicotine and acetylcholine on the isolated heart of *Cancer magister*. On the basis of this similarity, a hypothetical mediation mechanism was proposed for the decapod heart, and it was suggested that the effects of acetylcholine might be nicotine-like at the ganglion and muscarine-like at the neuro-muscular junction, since it had been demonstrated that the effects of acetylcholine are blocked by the administration of atropine (Welsh, 1939*a*, 1939*b*, Davenport, 1941). It became apparent that further tests with a number of sympathomimetic drugs and blocking agents would be of interest.

The technique of isolation and perfusion was similar to that previously used with one modification which should be mentioned, since it was found to give more uniform preparations. The heart was left attached by its ligaments to a polygonal piece of the carapace, which was clamped vertically in a fixed position; the posterior tip of the heart was tied off with a thread and attached to a light isotonic lever, and the perfusion fluid introduced via the aperture left by the removal of the descending vessel. In this way a minimum of manipulation was necessary after removal of the viscera.

The effects of the drugs that have been tested on the *Cancer* heart may be seen in the following table:

Drug	*Response*
acetylcholine	+
acetylcholine after eserine	++
atropine	O
acetylcholine after atropine	O
nicotine	+
nicotine after atropine	+
curare	O, −
acetylcholine after curare	O or freq. − / amp. +
muscarine	+
muscarine after atropine	O
doryl	+
doryl after atropine	+
mecholyl	+
mecholyl after atropine	O

+ = excitation, − = inhibition, O = no effect.

Previously it had been found, as observed in various arthropod hearts by other workers, that acetylcholine is excitatory, that treatment with eserine "sensitizes" the heart to acetylcholine presumably through its anticholinesterase activity, that atropine in low concentrations has little or no effect but that it abolishes response to acetylcholine. As has been stated, it had also been determined that nicotine has a marked excitatory effect on the heart resembling the effect of acetylcholine.

It next seemed of interest to determine whether atropine would have any effect on nicotine excitation. In a number of tests it was found that the response of the heart to nicotine 1: 50,000, a characteristic increase in amplitude and frequency, was in no way modified by previous treatment with atropine 1: 20,000 for twenty minutes, nor did atropine 1: 20,000, when introduced immediately after the response to nicotine appeared, in any way alter the response.

Effects of Curare

In several tests curare 1: 5,000 had no observable effect on either amplitude or frequency. A heart with a slightly irregular beat was regularized by treatment with curare 1: 400 and this regularization was accompanied by a decrease in frequency. Continued perfusion with curare brought about periodic stoppage in diastole (Fig. 1*A*). Carlson (1922) noted this cessation of rhythm in the crustacean heart after treatment with high concentrations of curare, but also observed a primary stimulation, which was not noted in the present tests. He states that after complete cessation of the rhythm, the heart remains excitable to direct stimulation.

Previous treatment with curare greatly affects the response to acetylcholine. A heart was perfused for approximately half an hour with curare 1: 5,000, which had no visible effect. Treatment after this time with acetylcholine 1: 10,000, a concentration which in the fresh heart produces a great increase in amplitude and frequency, can be seen (Fig. 1*B*) to have increased the amplitude only very slightly and the frequency not at all.

In a number of tests the characteristic effects of acetylcholine were not abolished but were profoundly modified by previous treatment with curare. A heart was treated for approximately twenty minutes with curare 1: 5,000. Introduction of acetylcholine 1: 100,000 brought about stoppage in diastole for a brief period. The curarization was then continued for a few minutes and acetylcholine 1: 10,000 introduced. A brief period in diastolic stoppage was followed by an amplitude effect

characteristic of acetylcholine, but by a marked decrease in frequency (Fig. 1*C*).

This is in marked contrast to the behavior of the vertebrate heart in which abolition or modification of the response to perfused acetylcholine or to vagal inhibition by previous curarization has not been reported.

In a single test in which a heart had been treated for thirty minutes with curare 1 : 5,000 and in which the response to acetylcholine had been all but abolished, nicotine 1 : 10,000 produced a marked increase in frequency and amplitude. The response to this concentration of nicotine was not as striking, however, as it would have been in a fresh heart.

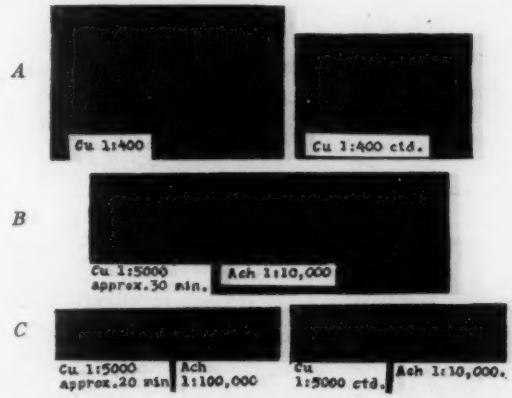

FIG. 1. *A*. Effect of curare 1 : 400. *B*. Effect of acetylcholine 1 : 10,000 after treatment with curare 1 : 5,000 for approximately 30 minutes. *C*. Effect of acetylcholine 1 : 100,000 and 1 : 10,000 after treatment with curare 1 : 5,000 for approximately 20 minutes.

Further tests should be run with this combination of drugs to determine whether curarization has an effect on the response of the heart to nicotine.

EFFECTS OF MUSCARINE

Concentrations of muscarine of 1 : 100,000 have a stimulatory effect on the heart of *Cancer* and increase frequency and amplitude without a rise in level of the recording. Concentrations as high as 1 : 5,000 (Fig. 2*A*) produce a marked increase in amplitude and frequency with an accompanying rise in level, but are apparently toxic, since slowing and irregularity of beat appear after perfusion for a short time. This is very similar to the effect of nicotine.

In a heart which had been treated with atropine 1 : 10,000 for fifteen minutes the response to muscarine 1 : 5,000 was completely abolished.

Effects of Doryl (Carbaminoylcholine)

Bonnet (1937) observed the effects of the sympathomimetics, doryl and mecholyl, on the heart of the crayfish while investigating the antagonism between strychnine and acetylcholine in these animals. Mecholyl was found to accelerate the crayfish heart while doryl in the concentrations used retarded it. Bonnet's results are, however, not strictly comparable to the present work since his drugs were applied directly to the heart *in situ* with nervous connections intact.

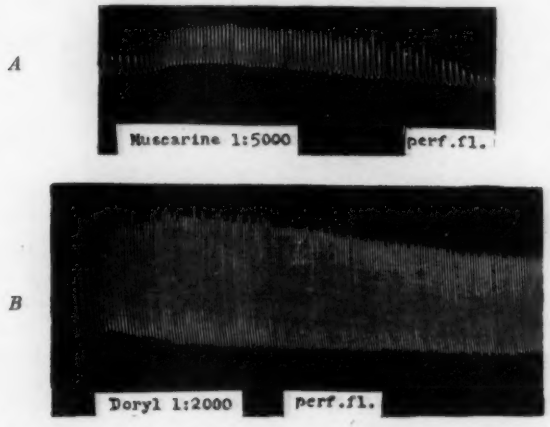

Fig. 2. *A*, Effect of muscarine 1 : 5,000. *B*. Effect of doryl 1 : 2,000.

In the heart of *Cancer* doryl 1 : 1,000,000 increases frequency and amplitude. This effect becomes more marked as higher concentrations are used. In no tests, however, even when concentrations as high as 1 : 2,000 were used (Fig. 2*B*) was it found possible to produce the marked increase in tone resulting in systolic tetany, characteristic of high concentrations of acetylcholine or nicotine.

In a number of tests it was found that previous treatment with atropine 1 : 10,000 had no effect on the response to high concentrations of doryl (1 : 2,000). In one test, however, a heart responded to doryl 1 : 1,000,000 with an increase in frequency and amplitude. Treatment with atropine 1 : 10,000 abolished this response when the doryl was

reintroduced, but failed to abolish the response of the heart to doryl 1:10,000.

Effects of Mecholyl (Acetyl-beta-methylcholine)

This drug also has a strong excitatory effect on the *Cancer* heart, but in a test with as high a concentration as 1:1,000 no rise in the level of the recording was observed.

In a heart which had responded to mecholyl with a great increase in frequency and amplitude, treatment with atropine 1:10,000 for fifteen minutes completely abolished any response when the mecholyl was reintroduced.

Discussion

Since the classical work of Carlson (1904) it has been thought by many that the heart-beat of arthropods in general may be initiated and controlled by the cardiac ganglion. This mechanism has been considered as a possibility in the heart of Crustacea by many workers. It has often been demonstrated that transsection of the ganglionic trunk immediately stops the decapod heart; great care must be taken in the introduction of a cannula for this reason.

A hypothetical mediation mechanism was proposed (Davenport, 1941) by which acetylcholine might have a nicotine-like action on the controlling ganglion and a muscarine-like action at the neuro-muscular junction. After an examination of the above experimental data, Dr. Welsh has suggested that in this mediation mechanism the modes of action of acetylcholine at the two points be reversed.

The effects of acetylcholine, nicotine, and muscarine on the beat of the heart are seen to be extremely similar. It is known that nicotine has as a primary action the stimulation of ganglion cells, and in the crab heart it apparently excites the intrinsic pacemaker. If acetylcholine and muscarine also excite the ganglion but via a muscarinic receptor-mechanism, then abolition of the effects of the two drugs by atropine may occur at this point. It would seem that mediation at the neuro-muscular junction beyond the spontaneously active ganglion may be by the nicotine-like action of acetylcholine, because atropine has no effect on the response of the heart to nicotine and because atropine alone has no effect on heart-beat. In no tests with atropine alone in low enough concentrations to be of physiological significance has it been possible to demonstrate any marked effect on rate or amplitude of contraction.

Curare abolishes the nicotine-like effects of acetylcholine but not its muscarine-like ones. The tests in which curare abolished beat altogether

are additional evidence that mediation at the neuro-muscular junction is by the nicotine-like action of acetylcholine. However, the tests in which low concentrations of curare abolished the excitatory effects of acetylcholine do not exactly agree with the hypothesis that mediation at the ganglion is by the muscarinic action. Under these conditions curare must prevent acetylcholine from altering the spontaneous activity of the ganglion, in which case the receptor-mechanism at that point would have to be considered both nicotine-like and muscarine-like. To fit in with the hypothesis curare should block the stimulatory effects of nicotine on heart-beat; unfortunately a single inconclusive test was run with these two drugs.

Doryl and mecholyl were tested to determine whether the heart would respond differently to a drug having both nicotine-like and muscarine-like actions than to one having muscarine-like alone. No marked difference in response could be demonstrated; however, it could be seen that the effects of doryl are less strongly antagonized by atropine than those of mecholyl, which are as in the vertebrates completely abolished.

It is apparent that studies must be made of the effects of drugs on the spontaneous activity of the intrinsic ganglion and the consequent behaviour of the heart.

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SOME NEUROHUMORAL EVIDENCE FOR DOUBLE INNERVATION OF XANTHOPHORES IN KILLIFISH (FUNDULUS)

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Many experimental investigations during the last dozen years indicate (see Parker's review, 1940*a*) that the dermal melanophores of several teleost fishes, including *Fundulus,* are subject to the influence of both pigment-dispersing and pigment-concentrating autonomic nerve fibers and that such influence is exerted through chemical mediators. The later contributions of Chin and Li (1941), Healey (1940), Parker (1940*b*), Parker (1941), and Parker and Rosenblueth (1941) in general strengthen the case both for double innervation of the effectors studied and for chemical transmission from the nerve endings to the melanophores.

Work on other chromatophores has remained relatively scanty. In the same kind of fish one would expect to find, as Parker (1932, 1940) has assumed for the xanthophores, the same type of nervous and humoral regulation as in the melanophores. Results reported by Smith and Smith (1935) and Parker (1937) on the squirrelfish and by Dalton and Goodrich (1937) on the paradise fish support this view as regards erythrophores. Double innervation of xanthophores is implicit in Abramowitz's (1936) consideration of the neurohumors that apparently govern them. I had found that both nerves, for pigment concentration, at least, and humoral substances control xanthophore responses in the killifish *Fundulus heteroclitus* (Fries, 1931). Giersberg (1932), using partly the same methods, could demonstrate pituitary but no nervous control of the xanthophores and erythrophores in the minnow *Phoxinus,* a form in which melanophores do receive nerves, probably both dispersing and concentrating. Vilter (1939) has held that the xanthophores in another cyprinid (*Carassius*) lack nervous regulation. The possibility that certain chromatophores in some species have *single* (pigment-concentrating) innervation is suggested by work on elasmobranchs (Parker, 1936).

The present paper [1] reports further results pertaining to the nature

[1] This paper owes its origin to a small project that had attracted the interest of Dr. G. H. Parker, but which he kindly ceded to me. The work was done during two early-September stays at the Marine Biological Laboratory, Woods Hole, Mass. To the helpful officers and personnel of that institution I acknowledge grateful indebtedness.

of the nervous mechanism earlier found to control the xanthophores in *Fundulus*.

Description of Experiments

The experiments consisted basically of caudal cuts similar to those Parker (1934) devised in studying catfish melanophores. The initial, or "primary," cut (1 in Fig. 1) severed two or three fin rays with their contained nerves, etc., and with their associated interradial tissue; this cut was made at or near the distal edge of the scale-covered zone or else just proximad of the main vascular arc connecting the axial caudal blood vessels with the mostly radially arranged peripheral ones of the fin. Distal to any such cut, a yellower as well as a darker band quickly appears, characterized by pigment dispersal in both yellow and black chromatophores (Fries, 1931).[2] After a week or more, when this primary band had faded by gradual pigment concentration in consequence of the fish's response to a white or pale-blue bottom, a pair of "secondary" cuts (2 and 2′ in Fig. 1) through an appropriate span of fin-ray branches established new yellow and dark denervated bands flanking the distal portion of the primary band. These primary and secondary cuts were carried out on males and females, about 6–8 cm. long, mainly of the striped killifish, *Fundulus majalis* Wahlb., which has more distinct xanthophores than the common killifish, *F. heteroclitus* L. Externally painted glass bowls (mostly 10-inch culture dishes) with running sea water served to contain the fish and to call forth their chromatic responses to colored backgrounds.[3]

In Parker's (1934) and Matsushita's (1938) experiments on catfish melanophores, pigment dispersal in secondary bands spread into previously faded primary bands but not into intact control bands; also, responses of denervated bands to black or white bottoms occurred by gradual spread from adjacent innervated areas. Blood-borne hormones being found innocent of such spread effects, it is held (1) that pigment-dispersing nerve fibers aroused to prolonged activity by the cut are responsible for the dispersal, and (2) that these nerves act by secreting a lipid-soluble dispersing "neurohumor" that can diffuse slowly even to rather distant melanophores.

Do the xanthophores of *Fundulus* behave similarly, calling for like interpretation? Among a group of 26 *F. majalis* given secondary cuts

[2] For an account of the disposition of the yellow pigment in the xanthophores of *Fundulus*, see Warren (1932).

[3] The paints used were Carmote Colorquic white (for some dishes turned just perceptibly bluish with a trace of prussian blue), the M. B. L.'s acid-resistant black, B. Moore & Co.'s Utilac medium blue and Utilac yellow.

after the fading of successful primaries, 15 showed indications, at least once, of some spread of the yellow effect produced in bands II and II′ into the I_d half of the primary band, yet not into the N areas (Fig. 1). Several of these afforded beautifully clear demonstrations. Among them were two given a third secondary cut (2_c in Fig. 1*B* and *C*) to provide an equal innervated band (N_{cd}) as simultaneous control; no trace of

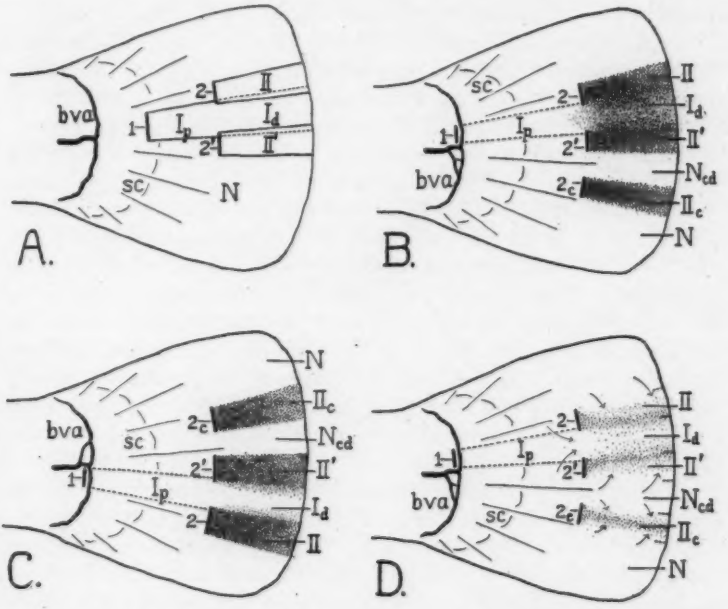

FIG. 1. Diagrams of *Fundulus* tail fin. *A*. Showing denervated bands produced by transverse incisions (all cutting radial blood vessels). *B*. Showing similar bands, but including one more for control, and showing primary incision that avoids blood vessels; with stippling to indicate a good example of pronounced affirmative results. *C*. Ditto; but stippled to indicate minimum affirmative results. *D*. Ditto; but stippled to indicate loss of pronounced effect (*B*) through general pigment concentration, arrows marking progress of fading of yellow zones, or encroachment of pigment concentration from innervated zones. 1, primary cut, and I, corresponding primary band, with proximal (I_p) and distal (I_d) portions distinguished; 2 and 2′, secondary cuts, and II and II′, corresponding secondary bands; N, normal innervated regions; 2_c, additional secondary (control) cut, and II_c, corresponding secondary band, providing N_{cd}, distal normal control band; bva, blood-vessel arc of main arteries and veins; sc, distal limit of scales. Cut 1 was made dorsal or ventral to the main axial caudal vessels according as collateral supply to the arc afforded best clearance; the position of II′ and the control bands was made to correspond inversely.

spread of the yellow effect from the adjoining bands (II′ and II_c) appeared in the control bands. In these two best cases and a few others the secondary cuts were made two weeks after the primary, by which time there was extra assurance of complete degeneration of the primary-band nerves. But, because as many as 11 of the 26 showed only negative or dubious, faintly affirmative results, I took the next opportunity to carry through experiments on another set of 25 *F. majalis,* each with the self-contained control afforded in band N_{cd} by cut 2_c (Fig. 1*B* and *C*).[4]

Sleek, sound fish were selected after at least two days of acclimatization to the laboratory aquaria, i.e., after the wave of deaths that in this species often follows seining and transport. Unlike the earlier lot, the fish were fed almost daily with minced clams. The unregulated temperature of the running sea water was close to 20° C.; occasionally the fish were exposed to warmer standing water. For all tail-cutting operations the fish were subjected to cold anesthesia in an ice-and-water mixture. The best tools for the cuts had proved to be chisel-like slivers, ¾–2 mm. wide, of a Gillette-type razor blade, fastened to handles several centimeters long. The cuts were made against an enlarged glass stage under the low power of a dissecting binocular microscope after removal of any scales covering the surface to be penetrated. To assure from the outset minimum vascular differences between the innervated regions and the primary denervated band, all primary cuts, usually through two or three fin-ray bases, were made just proximad of the blood-vessel arc and close dorsad or ventrad of the main axial vessels (Figs. 1*B* and *C*). Within the hour specific note was made in which rays and interrays the nerve supply was severed, as shown by dispersal in the chromatophores which was obvious in 10–30 minutes. Usually a recheck was made on the day after the cut. At this latter time the dark and yellow band was commonly less wide than originally. Some of this loss in width may be due to the eventual dominance of uncut concentrating fibers over the influence of less strictly radially distributed cut dispersing fibers (Mills, 1932). This narrower band after fading could be revealed as a pale band by putting the fish for a few minutes over a black bottom. It may then be considered fully denervated at least in regard to any dispersing fibers. The secondary cuts 2, 2′, and 2_c (Fig. 1) were made equally long, with the same chisel, carefully oriented to adjoin the previously recorded primary band and to make bands I_d and N_{cd} equally wide; they were made eight days after the primary cut, except for five sets made six days after and six sets made ten days after. All critical determinations

[4] Though I did not know of Matsushita's (1938) work when I first applied this method to three fish in my earlier lot of experiments, he had already devised and made use of the same expedient.

of the results in the primary and control bands were made with the binocular, the fish usually being held with bare fingers. The binocular was used, too, for most other observations of the state of the caudal xanthophores, and in every instance of possible doubt. Besides some daylight (two big windows, but dark interior), illumination was by two 75-watt daylight bulbs hung in white shades two and one-half feet above the bowls and four feet apart. Under these conditions the fish changed shade greatly in two minutes when shifted between black and white bottoms and showed definite macroscopic differentiation of dorsal yellow vs. not-yellow hues in 15–30 minutes on interchange of yellow and blue bowls.

The results for the second season resembled those of the first. Among the 25 fish, carried through to one or more observations following the secondary cuts, 14 yielded affirmative indications at least once of some spread of the pigment-dispersal effect from bands II and II′ to the xanthophores in I_d but not from II′ and II_c into N_{cd} (Figs. 1*B* and *C*); four more might be included in the same category except that the proximal part (I_p) of the primary band turned about as yellow as the distal part (I_d) when the secondaries (II and II′) were formed; in three others the indications were doubtful, and the remaining four gave only negative results in the time available. One of the 25 did not withstand the operations and handling well enough to be examined for the results more than once, and the condition of the tails of several others at the inspection time was below standard. None of these poor specimens was included among the definitely affirmative 14 cases. The good cases included at least two that demonstrated the spread effect, confined to I_d, as diagrammatically as Fig. 1*B*. In the others of the 14, the effect was less pronounced, in some consisting solely of a slight transition of the xanthophore state within the edges of I_d toward the degree of dispersal characterizing the xanthophores of the adjacent II and II′ bands (Fig. 1*C*).

Among the 14 fish were 9 that failed to give the same affirmative results at some other time or times of inspection. In this connection it is well to note that the yellow spread from bands II and II′ into band I_d is rarely visible in killifish kept over a bottom inducing full pigment concentration in the xanthophores; and when it does appear, it disappears on further manipulation of the fish. Accordingly, the full count of affirmative results in both sets of experiments was obtained only by coaxing, as it were. Such "coaxing" meant that when the primary bands had faded in the fish kept in white or blue bowls, yellow bowls were substituted to induce reduction of the factors for pigment concentration throughout the tail fin. Thereby dispersal was promoted, especially in the denervated zones, and above all in the freshly formed secondary bands.

As a rule, though contrary to the indications of the obvious yellowing of the body, the innervated xanthophores in the tail fin generally got no further than the intermediate state while newly denervated xanthophores regularly attained maximum or near-maximum pigment dispersal (maintained for days over a yellow bottom).[5] In case the innervated xanthophores did show too much dispersal, the fish were again placed in a white or blue bowl to induce some reconcentration in the innervated xanthophores for the sake of better contrast with the xanthophores in the secondary bands. Sometimes several shifts of the fish from color to color were necessary to obtain the balance between dispersing and concentrating factors in the primary band permitting demonstration of the spread into it of pigment dispersal excited in the flanking secondary bands. For their strong showing of the effect even my best examples depended upon favorable adjustment of bottom colors and of factors involved in handling or exciting the fish.

Discussion of Results

Considering the whole trend of results as tallied for the two lots of fish, the evidence seems favorable to the hypothesis that xanthophore innervation includes dispersing fibers that secrete a dispersing neurohumor capable of spreading from cell to cell. In two other corroborative experiments a single flanking secondary band resulted in a spread of its yellowing effect into the faded primary band but not into the equally contiguous innervated region.

The failure of the xanthophores in intact regions to be influenced by adjacent denervated bands and their ability, not shared by denervated xanthophores, to show *prompt* adaptive response to white or blue ground color confirms the conclusion (Fries, 1931) that the xanthophores are supplied with concentrating nerves. In addition, the results in general, and particularly in their dependence on the "coaxing" procedure, accord with the view that these concentrating fibers secrete a corresponding neurohumor which, like the dispersing neurohumor, can reach more distant xanthophores by diffusing through tissue lipids (cf. Parker, 1940*b*, on melanophores). Excess of such a concentrating neurohumor, richly

[5] That the yellow bowls generally failed even in the course of more than a day to evoke full or almost-full dispersal in the xanthophores of the tail fin was contrary to prior findings (Abramowitz, 1936, for this species), yet certainly typical of the fish in these experiments—irrespective of the year or of the food supply. All observations, with rare exceptions, were begun within a few seconds after the fish had been swimming undisturbed in the bowl. Thus there was too little time for excitement or other stimuli to induce much change in the xanthophores from the condition they had assumed. The incomplete response in the tail fin to the yellow bottom color remains unexplained.

produced in innervated parts of the tail during response to a white or blue bottom and spreading into the bands, may (especially if assisted by any substance carried in the blood) fully counteract the dispersing neurohumor everywhere except at the site of its active production and consequent greatest abundance in the secondary band. Thus applied, the hypothesis accounts for rather frequent negative results in the critical tests of cell-to-cell spread of a dispersing neurohumor.

Apparently less reconcilable with the hypothesis than simply negative results was the fact that increased yellowness (pigment dispersal), resembling the spread into the denervated band from bands II and II′, appeared in the control band (N_{cd}) once in each four of the group of 25 fish. Among the four were three of the 14 that on at least one other occasion showed the spread only in the denervated band (I_d). We are concerned, therefore, not with four fish versus 14, or four out of a total of 25, but with four single instances of a phenomenon that failed to appear in more than 60 opportunities. Local equilibria of several pigment-motor factors might get mutually out of step more readily under the shifting conditions of changing bottom colors and incidental handling than with steady visual stimulation from a white bottom. This possibility may offer an adequate explanation of the four instances of yellowing of the innervated control band.

There are further reasons for holding to the interpretations involving antagonistic nerves and neurohumors. In many instances the fading of a primary yellow band obviously proceeded axipetally, i.e., beginning at the longitudinal edges, gradually extending inward, and becoming complete last in the axis of the band. In several other instances, when the intact zones became yellow after a yellow band had faded, redispersal in the band, lagging behind the dispersal occurring elsewhere, clearly took place by invasion from the edges to the axis. I could find no evidence for supposing that differences in the flow of blood, carrying typical circulatory hormones, were responsible for this mode of xanthophore change in the denervated band. When the blood flow throughout the band (cut proximad of the blood-vessel arc) was equal, as far as discernible, to that in adjacent innervated regions, axipetal change occurred as much as when the flow was obviously obstructed in bands made by distal cuts. Therefore, Vilter's (1939) explanation of lagging, axipetal band changes in terms of defective band circulation, assumed to limit renewal of local stores of intermedin, would be untenable applied to the present results. Instead, the axipetal changes in the bands conform perfectly with the view that the phases of xanthophore response depend upon different chemical and physical equilibria of the medium, that antagonistic pigment-motor nerve secretions enter into such equilibria, and that slow cellular

transmission of such secretions from active fibers is the limiting factor of xanthophore response in regions where central control of the nerves is interrupted and where there is at the same time insufficient blood-borne pigmentary hormone to swamp the effect of the nerve secretions.

Both lots of killifish supplied many examples of progressive fading of yellow secondary bands inward from the innervated edge, while progressive fading occurred along the edge next the primary denervated band only considerably later, after any prior yellow spread from the secondary bands into the primary band had been dissipated, and through gradual roundabout invasion from the innervated zones (Fig. 1*D*). The dual neurohumoral explanation is again wholly adequate. In particular the fading of either secondary or primary bands inward from adjoining innervated sectors argues for nervous production and cell-to-cell diffusion of a concentrating neurohumor.

Pigment concentration in all areas where the xanthophores exhibited any dispersal took place repeatedly as I held fish on the microscope stage for one-half to three minutes. On such occasions yellow bands, especially older ones, faded considerably, if not completely, while I watched. Sometimes, when the first inspection was limited to a few seconds, another look ten minutes later revealed that the same sort of fading had occurred and had not yet been reversed during the interval back in the yellow bowl. Frequently correlated with such quick fading of yellow bands and with equally quick concentration in innervated xanthophores was simultaneous stoppage or near-stoppage of blood flow into all sectors of the caudal fin. Resumption of circulatory inflow in the next minutes with more or less distention of veins throughout the tail was correlated with redispersal in the xanthophores that had just shown concentration, the redispersal appearing more markedly in newer bands and in innervated zones. The fact that this redispersal appeared least in old denervated bands accords with the hypothesis of a dispersing nerve secretion and the idea that activity in dispersing fibers excited by cutting diminishes after a time as the nerves degenerate (cf. Parker, 1941). Once dominated by the concentrating agents and caused to fade, such older denervated bands could not, according to the hypothesis, so quickly come to contain as much dispersing neurohumor as could innervated zones or newly excited bands.

The quick pigment concentration in both denervated bands and in innervated regions seems, on the contrary, to depend on what the blood brings. It failed to occur equally just next to rather new cuts in the fin where the blood with less effective collateral circulation was more obstructed than distally in new bands. When the circulatory change happened to be noticeably unequal in different sectors of the fin, then the

first, fastest, or greatest concentration was several times observed to occur in the xanthophores of the sector supplied at the moment with the strongest blood flow. The facts noted make it improbable that the quick pigment concentrations are due to lack of oxygen in blood (cf. Fries, 1931; Parker, 1938), which has not been found to have such a quick effect. Suspicion falls more naturally on adrenaline: it is well known to have not only vasoconstrictor action but pigment-concentrating effects on some chromatophores, and Abramowitz (1936) found that injections of an adrenal preparation evoked the concentrated condition in both innervated and denervated xanthophores of *Fundulus majalis*.

A few experiments involving primary and secondary bands, duplicating those for *F. majalis*, gave comparable results for the xanthophores of *F. heteroclitus*. Out of seven fish given secondary bands 10–15 days after the primary cut, one showed a good, and two showed some, spread of yellowing from the secondary bands into the primary between them. In two more the primary band turned yellow even in its proximal part; in the other two the results were doubtful. As far as may be judged on this basis, the xanthophores of both species of *Fundulus* evidently have identical innervation.

Though the chief recent work on teleostean melanophores, supporting the hypothesis of double innervation and of neurohumoral transmission, deals with the catfish, various evidence indicates that the melanophores of *Fundulus heteroclitus* are under essentially the same kind of nervous control (Parker, 1940*a*). It ought, therefore, to be possible to demonstrate in *Fundulus*, though Parker found the catfish better for the purpose, that a new dark band induces an adjacent denervated pale region to darken, a change consonant with the idea of an invading dispersing neurohumor. In my experiments on *F. majalis*, while primarily concerned with the xanthophores, I observed the melanophores enough to gain a well-grounded impression that, in this species, they demonstrate this about as often as the xanthophores. Unquestionably the melanophores in the faded primary band, not in the pale innervated control band, in several cases showed marked pigment dispersal between strongly darkened, flanking, secondary bands, especially next to their margins. No indications of the same phenomenon could be detected in some other cases. If double innervation and neurohumoral transmission be accepted for the killifish's melanophores on wider grounds, then the resemblance between its melanophores and xanthophores is another point in favor of accepting both concepts for the xanthophores.

What of possible alternatives? The pituitary must be considered. That it does indeed supply a hormone important for dispersing the pigment in *Fundulus* xanthophores, as suggested by analogy with *Phoxinus*

and by the results of injecting pituitrin (Abramowitz, 1936), will be shown in another paper being prepared for publication. No dispersing hormone, however, can of itself account for the spread of dispersal from the secondary bands exclusively into the distal part of the test band when dispersal is elicited in the secondaries, nor for the failures of the spread to appear at all. On the other hand, if this hormonal mechanism, probably plus an antagonistic one (adrenaline?), is thought of as co-existing with the humorally mediated double innervation, then the previously noted exceptional occurrence of dispersal in I_p as well as I_d (Fig. 1) puts little strain on the neurohumoral interpretation; such exceptions might well occur when a surplus of pituitary hormone coincides with so reduced a supply of the concentrating nerve secretion that the latter, though remaining dominant in intact regions, fails to dominate in any part of the denervated bands. Also, if comparatively reactive chromatophores in such fishes as *Fundulus* are delicately poised in relation to three or more different controlling substances, considerable variability of response is to be expected, so that other deviations from the standard spread effect would follow from excessive variations in the blood supply of the hormones or in the tone of the pigment-motor nerves.

The most plausible alternative to the thesis advocating dispersing nerves would assume co-existence of the pituitary dispersing mechanism, and perhaps a concentrating hormonal mechanism, with single innervation supplying or withholding pigment-concentrating impulses. It implies appropriate flexibility of behavior in denervated and intact chromatophores. But even if amplified to include chemical transmission from the concentrating fibers (Veil, 1936), it accounts less well than double innervation for those changes in primary and secondary bands that occur by invasion from the edges. It fails still more to account for the re-eliciting of a yellow band by recutting an already denervated but faded band (Parker, unpublished; cf. Parker, 1940*b* and 1941).

Altogether the experimental results and incidental observations, added to prior data, in my opinion support the conclusion that the xanthophores of *Fundulus majalis* and probably also *F. heteroclitus* are governed by a neurohumorally mediated reflex mechanism, including dispersing and concentrating autonomic innervation, as well as a longer-distance humoral mechanism with a pituitary dispersing hormone (presumed to be intermedin) and probably some concentrating hormone.

Summary

In a majority of over 50 *Fundulus majalis* tested, the part of a previously faded primary denervated caudal band flanked by two new secondary bands showed pigment dispersal in its xanthophores, as if affected

by the stronger dispersal in those of the adjacent secondary bands. The effect appeared whether or not the radial blood vessels were cut by the incision that produced the primary band. Intact control bands, flanked identically, generally gave no sign of a like effect.

A few experiments on *F. heteroclitus* revealed comparable responses by its xanthophores.

These observations derived from the xanthophores were paralleled by some from the melanophores in *F. majalis*.

Characteristically, slow changes in the xanthophores in response to bottom color proceeded axipetally in primary bands, and they took place in secondary bands by an analogous invasion from the edge adjoining an innervated sector rather than from the edge of the primary band.

Quick pigment concentration apparently excited by handling, holding on the microscope stage, etc., appears in denervated bands as soon as in intact sectors.

These results, certain exceptions, and other available evidence together prompt the conclusion that the xanthophores of *Fundulus* are probably controlled by nervous and humoral mechanisms involving the following components in addition to a dispersing pituitary secretion and some concentrating hormone (adrenaline?): *A*. Double autonomic innervation, which includes (1) dispersing fibers, (2) concentrating fibers. *B*. Chemical mediation, the nerves secreting, respectively, (1) a dispersing neurohumor, (2) a concentrating neurohumor. *C*. Cellular transmission of these neurohumors to neighboring xanthophores.

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NOTES ON COLOR CHANGE AND PIGMENTARY INNERVATION IN A GOBY, A WRASSE, AND THE PLAICE

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Integumentary color change differs among teleosts. In many species it is more strictly a change of shade, while in some the hue is also actively altered. The effectors of all such changes, the chromatophores, appear to differ, according to their kind and the species in which they occur, in their mechanism of control. Recent work on teleost melanophores, going beyond the accepted conclusion that they are innervated, stresses the role of hormones (primitive, accessory, predominant, as the case may be), of double innervation in at least some species, and of chemical (neurohumoral) mediation of the nervous regulation. As for the erythrophores and xanthophores that may participate in color change, innervation seems established for some teleosts, not for others. A better unified understanding of these matters, amid so much apparent diversity, will depend on additional observation and experimentation.

To this end, exploratory study of several species of teleosts common near Fiskebäckskil[1] yielded results which, though in part incomplete or tentative, are recorded in this paper.

Methods

All the fishes were kept in running sea water, which stayed within two degrees of 17° C., and were fed every few days on minced mussels. Glass bowls, about 25 cm. in diameter, coated externally with black, white, bright rich yellow, pale blue, or vivid red paint were used to elicit the color responses. Ample daylight was admitted through large windows. All macroscopic determinations of color or adaptation were comparative, the fish being viewed simultaneously over like backgrounds, mostly white bowls or pale grayish sand. For microscopic examination of the chro-

[1] At the Kristineberg Zoological Station (of the Royal Swedish Academy of Science), on the west coast of Sweden. Accorded every facility and ready aid in supplying my wants, I owe Professor Einar Lönnberg, the director of the Station, and Dr. Gunnar Gustafson, the manager, particular acknowledgment of their hospitality.

matophores, chiefly in the tail fin, I used a dissecting binocular. Cracked ice in water served as anesthetic for the operations.

GOBIUS MINUTUS Pallas

This goby ("common goby") has plentiful red and small yellow chromatophores, besides melanophores and guanin-containing elements,[2] which on the trunk are commonly joined into chromatic complexes, as detailed by Ballowitz (1913*a*, 1913*b*). In the caudal fin occur prevailingly only separate red, yellow, and black pigment cells.

Meyer (1931) has described how this fish attains, rather slowly, a color well approximating that of several experimental backgrounds. My tests of its responses to black, white, red, and yellow essentially confirmed her results as regards the rate, nature, and extent of the adaptations to these colors, except that I found, in the tail fin, poorer differentiation of xanthophore response; their pigment was not concentrated much in response to red, as compared with the dispersal evoked by yellow, and usually did not, even in nine days, become completely concentrated in response to white. Meyer did not mention blue. I found that blue evokes a response distinct from those to red and yellow (besides black and white). For example, two lots of five and four fish in two days over blue and yellow, respectively, showed macroscopic differentiation, not only slightly of shade but clearly of hue. This difference was half lost in forty-five minutes over a neutral ground of sand and reversed, as verified microscopically in the tail, in six hours over the reversed colors, yellow and blue. The blue response involves pigment concentration in both erythrophores and xanthophores, and a more nearly intermediate condition in the melanophores. Were these the only factors, there would be no difference from Meyer's results with gray; but as the skin is chromatically more complex (e.g., iridocytes and iridosomal combinations are abundant on the trunk), the goby can turn truly bluish.

Morphological color change became obvious sooner than anticipated, especially in young fish less than 4 cm. long. After five days over white, considerable degeneration of melanophores and extrusion of melanin through the epidermis (cf. Odiorne, 1936) was seen to have occurred.

In response to changed bottom color, the melanophores usually changed twice as fast as the erythrophores, yet the latter could show concentration through more than half their full range in half an hour. The more vaguely outlined xanthophores seemed slower. Meyer (1931) was impressed by the slowness of complete concentration or dispersal of the pigment, even in the melanophores, and by the relatively quick start of

[2] Compare Odiorne's (1933) description of guanophores and discussion of the terms guanophore and iridocyte.

the change, even in the erythrophores. She supplied convincing evidence of the humoral control suggested by the slowness, but did not do likewise for the direct nervous control expected on the basis of earlier work on other teleosts (e.g., von Frisch, 1911 and 1912) and indicated by the initial rapidity of the change. Unqualified reliability of the latter indication seems open to question, however, for humorally activated pigment concentration in a denervated band may occur very quickly (Fries, 1942).

More direct evidence of nervous control is supplied by denervating operations in which two or three rays of the caudal fin with their interradial tissue were severed. In gobies kept in white bowls or over pale grayish sand, such cuts, whether at the distal border of the caudal scales, or more proximally, or out in the fin, regularly provoked slow pigment dispersal in the melanophores and erythrophores, and less obviously in the xanthophores, throughout a band distal to the cut and enclosing the cut rays and their branches. The dispersal appeared first in the melanophores, evidently reached the maximum for the erythrophores in about six hours, and took a little longer in the xanthophores. Subsequently the bands tended to fade, but in the case of 3-ray bands did so only slightly, although full concentration prevailed in the intact sectors in response to a light bottom (e.g., pale sand for ten days, then white for four). In the 2-ray bands, five days over sand plus five over white sufficed for the pigment of the melanophores and erythrophores to attain typically a concentration almost equal to that evoked in the intact parts of the fin. But in fish transferred to red for five days following five over sand, the red, yellow, and black pigments were dispersed to the same extent in such 2-ray bands as elsewhere in the fin. Moreover, when white-adapted fish with almost wholly faded bands were transferred to red for several days, the band pigments became redispersed to match the chromatophores in the intact regions. The redispersal in the band erythrophores and melanophores appeared in some of the fish to lag behind the dispersal outside the band. In case of the reverse transfer from red to white of fish with the band chromatophores in the same state as in other sectors, the resulting general concentration of pigment did proceed more slowly in the band. This lagging concentration in the denervated band was clear as regards both erythrophores and melanophores, but less sure as regards the xanthophores. The lag was verified in each of three fish tested as late as nineteen days after the denervating cut. The inability of the chromatophores in the caudal bands, despite fully restored circulation, to show normal concentration in response to visual stimulation is evidence that there is direct pigment-motor innervation involving concentrating nerves. The persisting capacity for some visually-induced response is evidence of humoral control.

Signs of beginning nerve regeneration in the denervated band were noted in a few gobies examined twenty days after the nerve section. By the twenty-eighth day no lag in the responses of the band chromatophores behind the innervated chromatophores could be detected, and virtually complete regeneration was further indicated in each of three gobies in which I recut the fin through the old scar with the result that a strongly renewed full-length band was displayed in six hours by all three types of chromatophores (cf. Parker and Porter, 1933, and Abramowitz, 1935).

Thus far there is close resemblance to the chromatophores of the killifish and the catfish. It suggests that the goby's melanophores and erythrophores, if not also the xanthophores, may have the same sort of neurohumorally mediated double innervation that is strongly indicated for the melanophores of *Ameiurus*. To throw some light on this possibility, I induced the formation of three secondary caudal bands, two flanking an old primary band and the third setting off an equivalent innervated band for control, as described in an earlier report on *Fundulus* (Fries, 1942). The secondary cuts were made two weeks after the primary, which presumably allowed quite ample time for complete nerve degeneration in the primary band. More or less convincing results appeared in the 11 fish in which the three secondary bands were successfully established. In the distal portion of the primary band (flanked by the secondary bands with more extreme pigment dispersal) the melanophores and erythrophores in all 11, and the xanthophores in two or more, showed a dispersal of their pigment that was not matched in the proximal portion of the primary band and not shown at all in the control band. So stated, the effect prompts explanation in terms of Parker's hypotheses—i.e., as due to a slow diffusive spread into adjacent regions of neurohumoral material secreted by recently cut and still excited dispersing nerves in the secondary bands and to the coincident dearth of concentrating factors in the denervated primary band as compared with the innervated control band. But the pigment concentration in the primary band was not fully equal to that in the innervated regions when the secondary cuts were made; and there was already a slight tendency in several cases to less concentration throughout the distal part of the fin. Still, it seemed safe to conclude that these two possibly misleading conditions were not responsible for all the effect observed.

Accepting the results, accordingly, as having some neurohumoral significance, one may ask why they should be taken as evidence for *dispersing* neurohumors and nerves: why not regard them rather as evidence of the assumption of a resting state (dispersal) by the chromatophores when their concentrating nerves are cut and when the supply of concentrating neurohumor diffusing into old bands from regions with uncut

nerves becomes exhausted in the part of a primary band isolated by the secondary bands? Rejection of this alternative stems first from other fish where fuller work supports belief in dispersing fibers (Parker and Rosenblueth, 1941) and in a different concept of chromatophore rest (Parker, 1940*b*). I would add an observation several times verified on *Gobius*. Handling pale fish provoked rapid darkening and reddening; i.e., the innervated caudal melanophores and erythrophores showed considerable dispersal of pigment in half a minute, while band chromatophores failed to show corresponding dispersal. This phenomenon can be explained better in terms of nerves than of hormones brought from a distant gland and supplied to a week-old band as richly as to the rest of the fin. It is better ascribed to the activity of dispersing nerves (cf. Parker, 1940*c*) than to inhibition of concentrating nerves.

When making this study of *Gobius minutus*, I was unaware of Vilter's related work (1938, 1939*a*, 1939*b*). Vilter obtained certain results with *G. lota*[3] and other teleosts that he took to be in conflict with Parker's reactivation of blanched bands, viz., he was unable to get renewal of pigment dispersal in faded denervated zones by recutting (Vilter, 1938). Besides the few already mentioned tests of nerve regeneration at three and four weeks, I recut the nearly faded primary band about midway of its length in two gobies. In both of them the primary band melanophores and erythrophores distal but not proximal to the new cut showed renewed pigment dispersal. Since the recutting was done fourteen days after the original cuts (Parker, 1941, Osborn, 1939), when degeneration of the original nerve endings was supposedly complete and regeneration could hardly have begun, the results are an incentive to further tests of the non-reactivation stressed by Vilter. His negative results seem of doubtful significance until confirmed in relation to other variables (Parker, 1941), such as the extent of interference with circulation. Vilter (1938) further observed that pigment dispersal was "permanent" in the middle one of three cut rays of the dorsal fin. My observation that 3-ray caudal bands fade less than 2-ray bands is comparable. Considered with due regard for the importance of intermedin as an additional factor for pigment dispersal, both observations are in harmony with those made on bands of various widths in *Fundulus* (Parker, 1940*a*, p. 183) and hardly contradict the hypothesis of competing nerve-secreted humors. Altogether, Vilter's results as published agree with the work of Parker and others on *Fundulus* in virtually all of many particulars, with the one noteworthy exception of the non-reactivation already discussed. Vilter's explanation is clouded by his assumption that xanthophore

[3] This species seems to be the same as *G. ophiocephalus* Pallas.

change is necessarily indicative of intermedin (Vilter, 1939*b*) and not subject also to direct nervous control such as is involved in *Fundulus* (Fries, 1942) and probably in *Gobius minutus*. Thus it appears premature to reject for *Gobius* the hypothesis that its melanophores, erythrophores, and possibly xanthophores are governed by dispersing innervation in addition to concentrating nerves and pituitary secretion.

Labrus ossifagus L. (L. mixtus Krøyer)

The literature of pigmentary physiology contains, so far as my search has revealed, no account of chromatic responses in this species, though there are references (Fuchs, 1914) to other members of the genus. Younger specimens and usually the females (Lönnberg and Gustafson, 1937) of this wrasse ("striped wrasse" or "red wrasse") are richly equipped with erythrophores but comparatively sparsely with melanophores, so much so that they are more or less old-rose in general tone. Both kinds of chromatophores occur fairly uniformly throughout the caudal fin; these are of moderate size and not very elaborate form. Smaller palish xanthophores are present also, but are irregularly distributed and in the tail fin are mostly grouped in blotchy patches.

Selecting the smallest specimens of the red phase available (15–20 cm. long), I tested their adaptive responses to colored bottoms by leaving groups of two each in the different colored bowls and then making macroscopic comparison and a quick microscopic inspection of the chromatophores in the tail fin.

An initial trial showed that running water was necessary. Kept an hour or more in the bowls without change of water, six fish were pale regardless of the ground color. The pigment concentration became obvious even in the iris, contrasting with its reddish, bright appearance in all healthy fish in running water, irrespective of the color of the bowl.

With water circulation provided, a distinct difference in shade appeared as response to dark and light bottom colors. Fish from the black bowl were darkest, those from the white, palest. As the range of difference was never wide, apparently not increased in periods longer than a day, the difference between the intermediately dark fish from the red bowl and those from the black or white was not striking, but it was verified on repetition and on interchange of pairs tested. On such interchange, the naked-eye effect induced by previous shorter or longer (even 2-day) stays over the given ground color reversed itself noticeably in three minutes and virtually completely in 15 minutes. These tests of themselves permit no conclusion regarding adaptation to hue as distinguished from shade. On the other hand, the caudal chromato-

phores, in attesting the reality of the response to shade, hinted at adaptation also as concerns hue. For example, all were practically in the concentrated state, on the average not quite punctate, in fish kept for two days over a white bottom. In those equally long over black, the melanin was fully dispersed and the red pigment also dispersed but not so widely; in those over red, the erythrophores showed more dispersal of pigment (though not extreme) than the melanophores, which were at most intermediate. Thus, the erythrophores and melanophores appear to respond differentially to a red as compared to a merely dark dish. Further tests with red vs. blue and red vs. yellow showed that the fish reacted differently to each of these three colors—becoming paler and less red over blue as against red, and paler and less red (in one case more yellowish) over yellow; and also darkening when shifted to red, but not when shifted to blue, from sand—but this study was not pursued far enough to verify decisively the apparent independence of erythrophore response to hue from melanophore response to shade.

The erythrophores resembled the melanophores in the reaction to handling and holding for microscopic inspection of the tail. In fish taken from white, dispersal of both pigments past the intermediate state occurred in half a minute or less. In fish taken from black, the contrary effect, general paling, ensued also very quickly. In no changes were the erythrophores conspicuously slower than the melanophores.

Innervated chromatophores of the body in some teleosts concentrate their pigment when chilled (Smith, 1928). This labrid on immersion in iced water quickly blanched, both melanophores and erythrophores over the body evidently exhibiting more intense concentration of pigment than observed under any other circumstances; but strong dispersal followed in 10–15 minutes (still iced), in fact became so complete in one fish, which then succumbed, as to obliterate the usually persistent whitish dorsal pattern spots.

In the case of another wrasse, *Crenilabrus*, with more striking changes of hue, von Frisch (1912) has shown that the rapidly reacting erythrophores have similar nervous control to that of the melanophores. Direct innervation of both types of chromatophores also in the red wrasse is suggested by the results of cutting a brace of caudal fin rays. At first the effects were mixed, the band distal to the cut turning dark red in only one of the five and seeming even blanched in one after 15 minutes. But after a day or two, by which time circulatory deficiencies were largely ameliorated, the band was distinct in two yellow-adapted, relatively pale fish, of four surviving, and became so in the other two red-adapted fish, when transfers to blue and to white induced pigment concentration in

the erythrophores and melanophores of all intact regions, those of the denervated sector remaining in the dispersed condition. After 15 days, mostly over a sand bottom but the last nine hours over blue, the band was still distinguishable, its erythrophores in a relatively dispersed state.

This evidence warrants no conclusion regarding the exact nature of the probably nervous control of melanophores and erythrophores in this wrasse. Yet no significant difference from the functioning of the probably doubly innervated melanophores of *Fundulus* has come to light.

Pleuronectes platessa L.

These notes are based on nearly 20 young specimens, 5.5–10 cm. long, of the European plaice. These fish had abundant small melanophores, about equally numerous xanthophores, of general distribution, and a few erythrophores, which, in the fins, were irregularly scattered. The layer of close-packed "iridocytes,"[4] in which Meyer (1931) found the other chromatophores embedded, is represented even in the fins: the xanthophores are generally encircled by pale gray, opaque cells, active participants in pigmentary change, that further obscure the characteristically ill-defined xanthophores.

In a test of adaptive response to true color (denied by Schaefer, 1921, who admitted only change of shade in this species), two young plaice kept in a blue bowl for four days acquired a distinctly different coloration from two kept for the same time in a yellow bowl, the former becoming bluish gray (cf. Mast, 1916, on other flatfishes), the latter yellowish; left in white bowls, the two lots retained a noticeable difference for some hours, but lost all of it by the next day. Blanching and darkening responses to white and to black bowls were typically less slow—noticeable in half an hour—but varied according to previous history. For example, long-sustained pallor (white bowl) markedly slowed adaptive darkening (melanin dispersal). This fact implies the accumulation of a concentrating substance during white adaptation (Meyer, 1931).

The response to yellow kindled hope of obtaining evidence regarding cellular transmission of a dispersing neurohumor for comparison with that obtained from the xanthophores of *Fundulus* (Fries, 1942). But the results of cutting fin rays were uncertain, if not negative. Yellow bands due to pigment dispersal in the xanthophores were never strongly differentiated and, partly because of persistent incomplete concentration in the uncut sectors, were often not discernible at all, though they were found in several favorable instances, beginning three or four hours after the cut. These slowly reactive xanthophores seem likely to be at least

[4] Again compare Odiorne (1933) rē guanophores and iridocytes.

predominantly under humoral control like that in *Phoxinus* (Giersburg, 1932).

The same experiments did, however, yield more significant data regarding the melanophores. Caudal bands (also dorsal-fin bands) from 2-ray or 3-ray cuts in pale or intermediate plaice regularly darkened, the melanophores showing dispersal, in less than an hour, excepting that in an instance of obvious circulatory stagnation the melanophores in the band assumed a fully concentrated state. The dark bands gradually faded in pale fish, full pigment concentration taking about two weeks in 3-ray bands (cf. Osborn, 1939).[5] Faded bands redarkened more slowly than intact sectors under adaptation to black. A 19-day-old band showing this lagging dispersal (except at its proximal extremity, where nerve regeneration seemed to have begun) afforded a good demonstration also of the change progressing axipetally, as if dependent on invasions of a dispersing neurohumor—the melanophores next inside the band from the intact regions characterized by full dispersal came to show nearly full dispersal before those in the band axis changed appreciably from the concentrated state.

Out of 12 fish in which flanking and control secondary caudal bands were successfully established, five gave definite, and two others gave uncertain, indications of dispersal in the melanophores of the distal part of the faded primary band adjacent to the secondaries. A similar clear effect was elicited in just two out of the same number of equivalent operations on the dorsal fin. The mixed nature of these results recalls the case of the xanthophores of *Fundulus* (Fries, 1942).

Some sort of direct nervous control is well-attested in various other flatfishes, and Ballowitz (1893) has supplied morphological, and Schaefer (1921) physiological, evidence of it in the plaice. In so far as the spread of pigment dispersal from secondary bands into an adjoining primary band may be considered acceptable evidence of the existence of dispersing nerves and their neurohumor in *Fundulus,* one may conclude—without prejudice to the possibilities of supplementary influence by an adrenaline-like factor in the blood (Osborn, 1939), by the pituitary hormone (cf. E. Meyer, 1931, Osborn, 1939, H. H. Meyer, 1939*a*), or by a retinal substance reported by Meyer (1939*b*) to cause paling in the plaice—that my results with the plaice point to similar dual nervous control for the melanophores in this species.

[5] As of interest in relation to the very long-maintained darkening of wide denervated bands observed by Osborn in *Pseudopleuronectes americanus,* it may be stated that 3-ray caudal bands in four *Pleuronectes flesus* (about 15 cm. long) failed to pale, except irregularly and along their margins, to match the pale innervated areas in the 26 days that I kept these flounders (over a gray-white bottom).

SUMMARY

Colored bowls evoked characteristic chromatophore responses in *Gobius minutus, Labrus ossifagus,* and *Pleuronectes platessa.* The responses, including those to blue and to yellow, effected differential adaptation of integumentary hue as well as shade, especially in the goby and the plaice. They confirmed that erythrophores, xanthophores, and melanophores may act independently, pigment dispersal in erythrophores and xanthophores depending on the color of the bottom while melanophores respond according to its darkness.

The changed chromatophore behavior resulting from fin cuts producing denervated bands indicated that the melanophores, erythrophores, and perhaps the xanthophores are innervated at least by concentrating fibers in the goby and that the erythrophores and melanophores are probably innervated in the wrasse.

Denervation experiments supplied evidence consistent with the hypothesis that dispersing as well as concentrating nerves and their neurohumors control the goby's melanophores and erythrophores and the plaice's melanophores. This evidence includes renewed pigment dispersal in the distal part of a faded, primary band where flanked by new, secondary bands (goby and plaice) and rapid dispersal in the not denervated melanophores and erythrophores when the fish were handled (goby).

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CONCERNING THE PIGMENTS OF THE TWO-SPOTTED OCTOPUS AND THE OPALESCENT SQUID [1]

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The majority of fishes and invertebrate species of the sea exhibit integumentary colors due either to melanin pigments or, especially in the invertebrates, to carotenoids. Carotenoids are often also relatively concentrated in such tissues as the liver or digestive gland, and in eggs. Chromoproteins, flavines, purines, and certain more rare pigmentary compounds are discussed by numerous writers (e.g. Verne, 1926).

This paper concerns the pigments encountered in various tissues of two cephalopods of Pacific waters, namely the two-spotted octopus, *Paroctopus bimaculatus,* and the common squid, *Loligo opalescens.* Cephalopods studied by Lönnberg (1935) and the two species investigated by us have revealed a striking general absence of carotenoids from the integument and gonads and only traces in the eyes, but there are considerable quantities of these yellow, orange, or red pigments in the "liver" [2] of *Paroctopus.* Melanins characterize the ink of these mollusks, and are present also in the iris and skin. Octopods show relatively large quantities of melanin in their highly specialized chromatophores, while certain squids exhibit very little in the integument, the pigment there often appearing light brown or reddish instead of grey or deep brown as in the former group. We encountered, in the pericardial glands of the octopus, small amounts of yellow flavines as well as a red water-soluble pigment of unknown identity in the kidneys.

Lonnberg (op. cit.), making preliminary studies of the carotenoids of three cephalopods, reports lutein in the eyes of *Sepiola scandica, Rossia macrosoma,* and *Eledone cirrosa.* He found no carotenoids in the eggs of *Rossia* or *Sepiola,* and only possible traces in the testes of *Eledone.* Extracts of *Rossia* eggs, however, gave a strong color reaction with $SbCl_3$, suggesting the possible presence of vitamin A or a similar com-

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[2] The term as employed here refers to the large red-brown, grey or greenish gland communicating by a pair of ducts with the alimentary canal at the junction of stomach and intestine. Actually the "liver" and "pancreas" are incorporated together, without a very sharp division, in the single gland.

pound. Carotenoids were not detected in the skin of *Sepiola* or *Rossia,* but were reported in the mantle of *Eledone.* Especially notable was the presence of quantities of a carotenoid resembling lutein in the liver of *Eledone.* The livers of the other two species were not given special study.

Escher-Desrivières, Lederer and Verrier (1938) cite older observations of Kruckenberg on the purple pigments in retinae of certain cephalopods. Studying the retinal pigments of *Sepia officinalis, Octopus vulgaris,* and *Eledone moschata,* these authors found certain chemical and spectroscopic similarities between the cephalopod eye-pigments and those of classical types. They found, however, that the cephalopod retinal purple was stable in light, and they failed to find A vitamins in the material. More recent investigations by Wald (1941) have demonstrated vitamin A and retinene in the retina of the squid *Loligo pealii,* but no traces of these or other carotenoids in other squid tissues. According to Wald, vertebrates and invertebrates alike use vitamins A in the eye. Failure by other investigators to identify these carotenoids in the eyes of squids and other invertebrates is attributed by Wald to the low capacity of such forms for storing these substances. Wald points out that the quantity of vitamin A remains constant in the squid's eye in light and darkness, and that it may not participate directly in the visual processes. He claims that the deep purple pigment of the squid retina, sometimes referred to as visual purple, is really "a photostable, alkali soluble probably melanoid pigment," acting as a light-screen for the retinal cells, but probably playing no role in vision proper.

Materials and Methods

Specimens of *Paroctopus bimaculatus* were taken freshly from the sea and killed by immersing in tap water just preceding analysis. Small specimens (about 6 inches in length) of the squid, *Loligo opalescens,* were bought from wholesale fish-dealers in San Diego. The animals showed no signs of decomposition, having been stored on ice since capture a few days earlier.

Brown or black melanin pigments were detected in the various tissues merely by visual inspection.

Flavines were characterized by their solubility in aqueous solutions, insolubility in organic solvents such as petroleum ether, bleaching of the yellow color by the addition of sodium hydrosulfite, reversible on shaking in air, and light absorption in the violet end of the spectrum near 417 mμ.

Carotenoids were extracted, adsorbed on chromatographic columns,

and given chemical and spectroscopic study in accordance with standard methods described elsewhere. (Fox and Pantin, 1941; Scheer, 1940; Fox and Scheer, 1941.)

Results

A preliminary survey, summarized in Table I, shows the distribution of certain of the pigments among various tissues of the two species, and reveals a few arresting facts. In *Paroctopus* we found no sexual dif-

Table I

Distribution of Pigments in the Tissues of *Paroctopus bimaculatus* and *Loligo opalescens*

Species	Tissue	Weight, grams	Carotenoids	Flavines	Melanins
Paroctopus bimaculatus; two specimens, male (742 gm.) and female (778 gm.).	Salivary glands	♂ 3.8	–	–	?
		♀ 3.9	–	–	?
	Crop, stomach and intestine	♂ 4.2	traces	–	–
		♀ 6.4	traces	–	–
	Gills	♂ 6.4	traces	–	–
		♀ 9.1	traces	–	–
	Liver	♂ 13.8	+++	–	–
		♀ 20.1	+++	–	–
	Ink	1 to 2	+	–	+++
	Eyes	♂ 2.1	traces	–	+
		♀ 2.7	traces	–	+
	Gonads	♂ 13.2	traces	–	–
		♀ 2.3	–	–	–
	Pericardial glands	♂ 2.5	–	+	–
		♀ 2.4	–	+	–
	Kidneys*	♂ 1.3	–	...	–
		♀ 1.2	–	..	–
	Heart	♂ 1.3	–	–	–
		♀ 1.3	–	–	–
	Muscle and skin†	♂640	–	?	skin +
		♀709	–		muscle –
	Eggs‡	33.8	traces‡	?	–
Loligo opalescens 7 ♂, 5 ♀	Gills		–	–	–
	Digestive tract		–	–	–
	Ink and sac	2.8 (from 12 animals)	trace?	–	+++
	Eyes	16.5 (from 12 animals)	(+)	?	+
	Accessory nidimental gland	0.9 (from 12 animals)	traces	–	–
	Rest of body (including gonads)		traces?	.....	traces

* The organs, purple in color, yielded quantities of a water-soluble, blood-red pigment discussed in text.

† Acetone extracts of muscle + skin gradually turned a pink-orange color, as did the ground tissue tself. This was not due to either carotenoids or flavines. The pigment was unchanged by acids or salts, but turned yellow in base, and its acetone solution gradually bleached in air.

‡ Laid by another female. Traces of yellow pigment soluble in petroleum ether or $CHCl_3$, gave pink to mauve color in $CHCl_3$ with added $SbCl_3$.

ferences in pigmentation. Here, as in *Loligo,* neither the mature testis nor the ovary or deposited eggs showed more than possible traces of carotenoids. The same was true in gills, digestive tract (minus liver) and eyes, while in the other tissues these pigments were absent. Carotenoids were yielded in considerable quantities by the relatively large liver, however, and were present in appreciable amounts in the ink stored in its sac, attached to the liver but not in direct communication with it. Flavines

were extracted only from the yellowish tripartite pericardial glands (of excretory function), while melanins characterized the ink, iris and skin.

The kidneys of *Paroctopus* were deep purple-red in color and when triturated yielded a blood-red water-soluble pigment. This pigment is insoluble in acetone or alcohol, being reversibly precipitated from water by these solvents. It contains iron and possesses reducing properties. Study of this pigment is being continued, and a more complete account of it will be published later.

Loligo was found to exhibit but little pigment of any kind in most of its tissues. Neither of the mature sexes yielded any pigments in the gonads. Flavines were not detected with certainty, and carotenoids were present in only very slight quantities in the eyes, while the other tissues yielded none or only suspected traces. While the body integument contained very little melanin this pigment was present in both iris and ink in considerable quantities.

Carotenoids in the Liver and in the Ink of the Octopus

The carotenoids were classified according to (1) their behavior in the partition test, both before and after hydrolytic treatment with alcoholic potassum hydroxide: carotenes and xanthophyll esters remain in the upper, or epiphasic layer of petroleum ether, while free xanthophylls migrate to the lower or hypophasic layer of 90 per cent methanol; following hydrolysis, carotenes remain unchanged, xanthophyll esters from the epiphase are split to give free xanthophylls which behave accordingly, while free carotenoid acids are now removed as potassium salts; (2) carotenes were separable one from the other by adsorption on Tswett chromatographic columns of $Ca(OH)_2$, while xanthophylls were resolved into individual pigments on similar columns of $CaCO_3$; (3) finally, absorption spectra of the separate pigments were determined with a Hartridge Reversion Spectroscope or with a Bausch and Lomb Spectrophotometer. Carbon disulfide was employed as solvent in all of the carotenoid observations.

Liver

Carotenes, represented by from two to five colored zones on the chromatogram of the hydrolyzed epiphase, usually showed a fairly prominent red or orange zone near the top, and a yellow zone nearer the bottom of the column. One of the more prominent upper zones gave absorption maxima agreeing closely with those of β-carotene, i.e., 519.5 mμ and 484.4 mμ (Hartridge) and 510 to 512.5 mμ and 482.5 to 485 mμ (B. and

L. spectrophotometer). Another prominent carotene-like pigment appeared above the β-carotene zone, with absorption maxima at from 505 to 507.5 mμ and 480 mμ. At times the shorter wave-length maximum had higher values of 484 to 486 mμ. We did not identify this pigment with any known carotene.

Xanthophylls, present both free and as esters, were represented by considerable quantities of lutein (505 to 508 μ and 474.5 to 479 mμ by Hartridge; 505 to 510 mμ and 477.5 mμ by spectrophotometer), and by lesser amounts of other pigments of the taraxanthin or eloxanthin range of spectra (500 to 505 mμ and 469 to 473 mμ); occasionally a third type was encountered, below the other two on the chromatogram, with absorption maxima not unlike those of antheraxanthin or petaloxanthin, i.e., 512 mμ and 481 mμ (cf. Strain, 1938).

Acidic carotenoids, appearing only after hydrolytic treatment, occurred in both the epiphase and hypophase.[8] They always presented a single, broad and asymmetrical maximum, difficult to determine with precision in the Hartridge instrument, but giving thereby values from 501 to 507 mμ, and, with the spectrophotometer, a broad hump between the values of 500 to 507 mμ, with a slight maximum at about 503 mμ.

Ink

Carotenes were absent.

Xanthophylls, both free and esterified, were found in smaller quantities than in liver tissue. Hydrolytic treatment did not permit subsequent recovery of sufficient quantities for critical spectroscopic study. Inspection of the fresh ink extract, however, revealed, in one instance, absorption maxima similar to those of lutein (510 mμ and 475 mμ). The epiphasic pigments of the ink carotenoids dissolved in carbon disulphide gave an orange solution of esters with maxima at 502.6 mμ and 472 mμ (cf. eloxanthin), while the hypophasic pigments in carbon disulfide failed to yield sharp maxima. Hydrolysis of the epiphase yielded free xanthophylls accompanied by the familiar carotenoid acid. The hypophase yielded, on hydrolytic treatment, an acidic carotenoid, sometimes but not always accompanied by a non-acidic xanthophyll.

In Table II are presented data concerning the relative mass of liver tissue and the range of concentrations of carotenoids encountered in liver and ink. Since the preponderant carotenoid of both liver and ink exhibited, in chemical, adsorptive, and spectroscopic behavior, properties agreeing closely with those of lutein, the concentrations of mixed carotenoids were measured, for purposes of comparison, in "lutein equivalents" with a Bausch and Lomb visual spectrophotometer.

[8] Always hypophasic after hydrolysis of the extract.

The combined weight of liver and ink is seen to vary between 1.86 per cent and 3.29 per cent of the total body weight, with a mean value of about 2.7 per cent. We were rarely able to recover more than one or two grams of the ink, which represented from 5 to 11 per cent of the combined weight of liver, ink-sac and ink.

The concentration of carotenoids (in lutein equivalents) in liver tissues ranged between 1.2 and 8.3 mg. per 100 gm. with a mean value of about 3.5, in freshly excised tissue from normal animals, i.e., excluding the relatively low value of 0.8 given by the autolyzed liver of animal No. 7. The ink of freshly caught specimens yielded appreciable amounts of

TABLE II

Concentrations of Carotenoids in Liver and Ink

Animal (sex)	Weight, grams	Liver + ink weight, grams	Liver and ink % of total body weight	Total carotenoids (as lutein) mg./100 g.
1 ♂	742	13.8	1.86	2.4
2 ♀	778	20.1	2.58	2.1
3 ♂	716	20.6 (liver)	3.19	1.2
		2.3 (ink)		0.70
4 ♂	721	18.9 (liver)	2.77	3.9
		1.1 (ink)		0.55
5 ♂ (starved)	820	25.4 (liver)	3.29	.....[1]
		1.6 (ink)		0.0
6 ♂ (immature)	493	12.7	2.57	8.3[2]
7 ♂	623	15.1	2.42	0.82[3]

[1] Very little of the pigment in this specimen was carotenoid, having evidently been altered by starvation.

[2] Quantity of carotenoids unchanged by incubating toluene-preserved macerated liver at its natural pH (5.42) for 44 hours at 37° to 40° C.

[3] Liver + ink autolyzed by placing in closed flask in presence of a few drops of $CHCl_3$ for 16 days at room temperature.

carotenoids showing values of from 0.55 to 0.7 mg. per 100 gm. (mean value from two determinations 0.62 mg. per 100 gram) while the ink of one specimen (No. 5) which had been starved for two or three weeks was found to be completely lacking in carotenoids.

DISCUSSION

Regarding the chief role of the liver-pancreas in cephalopods, Jordan (1929) states that it possesses only a secretory function, elaborating digestive enzymes which are passed into the gut during feeding.

In its lack of absorptive function, and its secretion of extracellular enzymes, the liver of cephalopods differs markedly from that of lamellibranch mollusks (see Yonge, 1931). The California mussel, a typical

phytoplankton feeder, passes great amounts of minute plant plastids into its digestive diverticulum, or "liver," whence certain cells engulf and absorb food, passing the undigested residues back into the digestive tract (Fox et al., 1936; Scheer, 1941, and unpublished observations on voiding of feces during early starvation). It would appear obvious that the liver of the mussel receives its rich supply of carotenoid pigments in a direct way from the plant material taken in. The liver of the octopus, on the other hand, probably receives its supply of carotenoids through the blood stream.

Very little seems to be known of the physiology of ink secretion in cephalopods, or of the biochemistry of the ink itself, beyond its rich melanin content. Its carotenoid content provokes much thought. We are aware of the loss of carotenoids from ectoderm in various ways, such as the sloughing of skin, the growth of feathers in certain birds, the discharge of ear-wax in cattle, and the presence of such pigments in the cores of solid materials secreted from the femoral pits of iguanas (unpublished data). Save for the storage of carotenoids in egg-yolk of many oviparous vertebrates and invertebrates, and the secretion of such pigments in the milk fat from mammary glands, the discharge of carotenoids from an internal structure to the outside would appear to be rather unique.

Carotenoids of *Paroctopus* disappeared from both liver and ink in a starved specimen, yet were reduced in amount only very slowly on autolysis of the liver at room temperature, or by incubation of the ground tissue at 37° C.

Clearly, a thorough investigation of the physiological role of the liver is called for, likewise a biochemical study of the ink and its relationship to the metabolism of the animal as a whole, with particular reference to the liver.

Summary

The kinds and tissue-distribution of various pigments in the cephalopods *Paroctopus bimaculatus* and *Loligo opalescens* are described and discussed. Melanins, stored in quantity in eyes and ink of both species, are far more plentiful in the integument of the octopus than in that of the squid.

Flavines were detected with certainty only in the pericardial (excretory) glands of the octopus. This species also yielded, from its kidneys, a water-soluble, iron-containing red pigment with reducing properties.

Carotenoids were present only in traces in any of the organs of the squid. This was likewise true of the octopus, save in the liver-pancreas of this species, which contained a variety of carotenoids in amounts of

3.5 mg. (lutein equivalents) per 100 grams of moist tissue (average value). Ink from the sac adjacent to the liver contained 0.55 to 0.70 mg. lutein equivalents per 100 grams.

In the liver of *Paroctopus,* free and esterified xanthophylls, predominantly of the lutein class, were accompanied by smaller amounts of other xanthophylls, β-carotene and an additional unfamiliar carotene, and a unique carotenoid acid, appearing on hydrolysis, not identical spectroscopically with astacene or with the metridene of Fox and Pantin.

The ink of this species yielded no carotenes, but free and esterified xanthophylls and (on hydrolysis) a carotenoid acid similar to respective compounds recovered from the liver. Whilst starvation brought about the gradual disappearance of carotenoids from liver and ink, autolysis of the liver or incubation of its ground tissues resulted in only slow loss of the pigments.

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STUDIES ON DEROPRISTIS INFLATA (MOLIN), ITS LIFE HISTORY AND AFFINITIES TO TREMATODES OF THE FAMILY ACANTHOCOLPIDAE

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INTRODUCTION

During the summer of 1939, three undescribed species of cercariae, one of which was a modified trichocercous larva, were found in the marine snail, *Bittium alternatum*, from Waquoit Bay, Cape Cod, Massachusetts. This trichocercous cercaria penetrated and encysted in *Nereis virens* and this annelid, when collected in the vicinity of infected snails, was found to be naturally infected. Of the trematodes that had been found in an extended survey of fish parasites in the vicinity, only two species could be regarded as possibly the adult of the cercaria in question; these were *Deropristis inflata*, belonging to the family Acanthocolpidae, and *Homalometron pallidum*, a species allocated to the subfamily Anallocreadiinae, family Allocreadiidae. Of the two, *H. pallidum* was at first believed most likely to be the adult of the cercaria from *B. alternatum*, since Hopkins (1937) had described remarkably similar cercariae for *Anallocreadium* and *Microcreadium*, both anallocreadiine genera closely related to *Homalometron*. This belief was supported by a comparison of the present cercaria with the adult of *H. pallidum* which showed that they agreed in possessing eye-spot pigment, a short, sac-shaped excretory vesicle, ciliated main excretory tubules reaching only to the ventral sucker, and similar branching of secondary tubules.

Each new life history study provides increasing evidence that trematodes properly allocated to the same family have life cycles involving similar embryological and larval stages and usually intermediate hosts that are closely related. It seemed unlikely, therefore, that *D. inflata*, a member of the family Acanthocolpidae, would have a trichocercous cercaria that encysted in annelids, since Martin (1939) had shown that another acanthocolpid, *Stephanostomum tenue*, has an ophthalmoxiphidiocercaria which encysts in fishes. Furthermore, metacercariae of at least

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two other genera in the family are known to occur in fishes. Although Carrère (1937, 1938) had identified a metacercaria from *Nereis dumerilli* as that of *D. inflata,* his observations did not seem at first to be significant for the present study since a number of cercariae encyst in species of *Nereis* and the most variable phase of the trematode cycle is that enabling the parasite to reach the definitive host. Feeding experiments, however, have demonstrated conclusively that the cercaria from *B. alternatum* is that of *D. inflata* and that the family Acanthocolpidae, as constituted at present, is not a natural group of closely related trematodes.

Although numerous species have been described and allocated to certain acanthocolpid genera, particularly *Stephanostomum* (here regarded as taking precedence over *Stephanochasmus* in accordance with the recommendation in Article 36 of the Rules of Nomenclature), a critical analysis of the family has been prevented by a lack of information concerning the type genus *Acanthocolpus,* and particularly the excretory system and life histories of acanthocolpids. The imperfectly described *Acanthocolpus liodorus* was the only known representative of the type genus until Srivastava (1939) described three new species of *Acanthocolpus* from Indian marine fishes. A better understanding of the type genus, and knowledge of the life histories and excretory systems of *Stephanostomum tenue* and *Deropristis inflata,* afford an opportunity to analyze the interrelationships of the acanthocolpid trematodes and their affinities to other groups.

Historical Review

Ward (1938) has reviewed the family Acanthocolpidae and the genus *Deropristis,* supplementing previous descriptions of the two known species, *D. inflata* (Molin) and *D. hispida* (Albildgaard). References concerning these species may be obtained from Ward's paper and need not be repeated here. A brief review of the family is desirable as a basis for discussion later.

The first attempt to group the trematodes at present allocated to the family Acanthocolpidae was made by Lühe (1906) who proposed the subfamily Acanthocolpinae (family unmentioned) to contain the genera *Acanthocolpus, Stephanochasmus* (syn. *Stephanostomum*), and *Deropristis,* and the species *Distomum semiarmatum* Molin. Later, Lühe (1909) established the family Acanthocolpidae, apparently to contain all these forms although he mentioned only *Deropristis inflata, D. hispida,* and *Distomum semiarmatum;* these species occur in migratory fishes and Lühe was concerned with only freshwater forms. He did not propose a subfamily Acanthocolpinae at that time, as assumed by some authors,

but stated that the various genera apparently belonged to several subfamilies. Odhner (1911), evidently disregarding Lühe's proposal, stated that *Stephanochasmus, Dihemistephanus, Acanthopsolus,* and *Distomum osculatum* Looss constituted a distinct family but did not propose a name for it. Nicoll (1913) erected the genus *Lepidauchen* which seemed to him to be an intermediate form combining the characters of the genera *Lepocreadium* and *Stephanochasmus*. He later (1915) placed *Lepidauchen* in the Lepocreadiinae, *Stephanochasmus* and *Acanthopsolus* in the Stephanochasminae, all of the family Allocreadiidae, and *Deropristis* in a list of forms of uncertain position. Poche (1925) criticized Nicoll's allocation of *Stephanochasmus* and *Acanthopsolus* to the family Allocreadiidae, removing these genera to the family Acanthocolpidae, to which he added *Dihemistephanus, Acanthopsolus,* and the new genus *Tormopsolus*. He also followed Pratt (1916) in reducing *Lechradena* to synonymy with *Stephanochasmus* and Odhner (1911) in the opinion that *Neophasis* is synonymous with *Acanthopsolus*. To include a new genus, *Echinostephanus,* Yamaguti (1934) redefined the subfamily Stephanochasminae, placing it in the family Acanthocolpidae. Ward (1938) denied the validity of both the genus *Echinostephanus* and the subfamily Stephanochasminae, but proposed a new subfamily, Acanthopsolinae, for the single genus *Acanthopsolus*.

Materials and Methods

During the summers 1939–41, several thousand *Bittium alternatum* were examined for larval trematodes. The snails were collected from Waquoit Bay at Menauhant, Massachusetts, and the infected specimens were separated by isolation to provide an abundant supply of cercariae. To obtain metacercariae of known age, a specimen of *Nereis* was placed in a large crystallizing dish and exposed to all the cercariae that emerged from a number of infected snails during a 24-hour period. After exposure, each annelid was kept either in a finger bowl half filled with sand, covered with cheesecloth, and placed in running sea water, or in a dish of water which was aerated without changing. Various species of fish for feeding experiments were isolated in the laboratory for at least three weeks before use. The experimental animals were killed and examined from one to seven days after being fed infected annelids. If, kept for longer periods, the worms either failed to establish themselves in unsuitable hosts such as *Fundulus* and flounders, or the eel, the natural host, they developed until they could not be distinguished with certainty from worms that might have been present when the fish was collected.

Throughout the study, living material was used extensively although

measurements were taken mostly from recently killed or fixed and stained specimens. Since the living cercaria is extremely active and difficult to measure without considerable coverglass pressure, measurements were taken mostly from larvae that were killed by placing them on a slide in a large drop of water, covering and holding over a flame for a moment. When treated in this manner, practically every cercaria died in a moderately extended, straightened position, and the body did not become opaque as when killed with the usual fixatives.

Neutral red and Nile blue sulfate were used supravitally and permanent whole mounts were stained with paracarmine. All measurements are in millimeters and all figures, except those indicated as free-hand drawings, were made with the aid of a camera lucida.

We wish to express our thanks to Dr. H. W. Manter for the privilege of examining material of *Dihemistephanus brachyderus* and to Professor H. W. Stunkard for helpful suggestions and criticism during the preparation of the manuscript.

Observations

Proof of the Life History of Deropristis inflata

Since at first we suspected that the cercaria was the larva of *Homalometron pallidum,* both naturally and experimentally infected annelids were fed to four *Fundulus heteroclitus,* a species which serves as the natural host of that trematode. These fish were examined five days later and found to be uninfected. Next, a large *Nereis,* heavily infected with 14-day metacercariae, was fed to a summer flounder, *Paralichthys dentatus,* which had been isolated for four weeks and had begun to take food. When killed and examined the following day, several hundred recently excysted worms were recovered from the intestine. These worms were all the same size and showed the conspicuous spines of the dorsal hump and lateral forebody characteristic of *Deropristis.* This observation was made too late to conduct further experiments until the following summer (1941) when the study was continued and the feeding of experimentally infected annelids to eels yielded large numbers of immature *Deropristis.* Control fish were either negative for *D. inflata* or harbored only specimens in an advanced stage of development. A careful study of the metacercaria revealed that the peculiar spination of *Deropristis* was just beginning to differentiate, the dorsal and lateral spines being distinctly larger than adjacent body spines.

Finally, a careful comparison of the excretory system of the cercaria and that of the young adult in both natural and experimental infections showed complete agreement. Thus the experimental results

and morphological evidence afford conclusive proof of the life history of *D. inflata.*

Description of stages in the life history of Deropristis inflata
Adult (Figs. 1–5)

The common eel, *Anguilla rostrata,* serves as the natural definitive host of *D. inflata* in the Woods Hole region. Practically every eel examined, except those that had been in captivity for several weeks, was found to be infected. The worms occurred throughout the intestine, being more numerous toward its posterior end.

The fairly complete accounts given by Odhner (1902) and Linton (1940) and supplementary observations by Manter (1926) and Ward (1938) make it unnecessary to give a complete redescription of *D. inflata.* Measurements of our specimens are well within the limits given in other descriptions. General morphological features are shown in Figs. 1–4. Since extensive observations have been made on living specimens, it is desirable to supplement existing descriptions of body spination and the reproductive and excretory systems.

EXPLANATION OF PLATE I

(All figures concern *Deropristis inflata*)

FIG. 1. Mature adult, ventral aspect.
FIG. 2. Spines of (A) metraterm and (B) cirrus (free-hand).
FIG. 3. Adult. Dorsal aspect of anterior end showing details of spination (many small spines omitted).
FIG. 4. Adult. Lateral view showing details of spination and terminal genitalia (many small spines omitted).
FIG. 5. Female reproductive system, dorsal view (free-hand).
FIG. 6. Embryonated egg from terminal portion of uterus.

ABBREVIATIONS

CI, cirrus.
CS, cirrus sac.
DV, common vitelline duct.
EG, egg.
EP, excretory pore.
ES, esophagus.
EV, excretory vesicle.
EY, eye-spot pigment.
GA, genital atrium or sinus.
GL, cervical gland.
GP, genital pore.
IN, intestine.
LC, Laurer's canal.
LV, left vas efferens.
ME, metraterm.
MG, Mehlis' gland.
OD, oviduct.
OS, oral sucker.
OT, oötype.
OV, ovary.
PH, pharynx.
PR, prostate cells.
RV, right vas efferens.
SC, cervical spines.
SD, dorsal hump spines.
SR, seminal receptacle.
SV, seminal vesicle.
TE, testes.
TV, transverse vitelline duct.
UT, uterus.
VD, vas deferens.
VI, vitellaria.
VR, vitelline reservoir.
VS, ventral sucker.

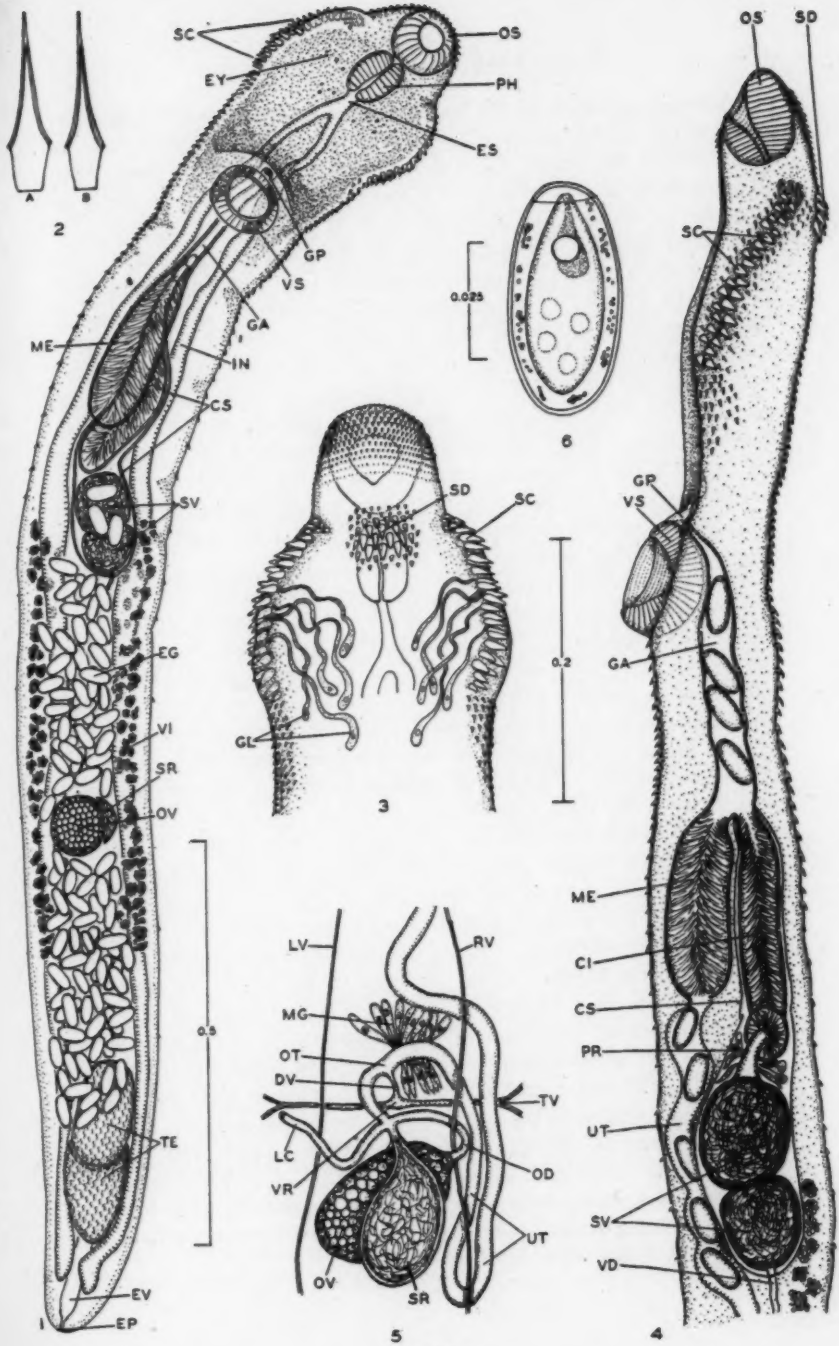
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PLATE I

Although spination of the body has been described rather fully, certain features have apparently been overlooked. The first three rows of spines behind the ventral lip of the oral sucker are slightly larger than those posterior to this level and the concave ventral surface of the body is devoid of spines from about the level of the cervical expansions to the ventral sucker. The large spines of the dorsal hump (Fig. 3) and probably also those of the cervical expansions (Fig. 4) develop at first as members of rows of spines quincuncially arranged. The number of enlarged spines on the dorsal hump is variable, but in very young worms there are usually ten in transverse rows of three, four and three. Adjacent spines are more or less enlarged in mature worms and the large spines become very prominent, more or less masking the original quincuncial pattern. They may become worn with age or even lost, giving the dorsal hump a ragged appearance. In living specimens, numerous conspicuous glands in the forebody are seen to open among the prominent spines of the cervical expansions. The ducts of a few such glands also open on the ventral surface of the forebody.

Living *D. inflata* is extremely favorable material for observing the reproductive system, the details of which can be observed completely in almost every specimen. The oviduct (Fig. 5) extends from the right side of the ovary for a short distance and then turns dorsally and to the left where its junction with the duct of the seminal receptacle, Laurer's canal, and the oötype forms a cross. From this junction, Laurer's canal extends to the left, opening at the dorsal surface considerably to the left of the median line. The oötype extends forward a short distance and then turns to the right, immediately receiving the duct of the vitelline reservoir. Just beyond this point, the oötype is surrounded by a moderately developed Mehlis' gland. From the oötype, the uterus extends posteriorly to the right of the ovary and returns on the same side. In young worms, this posterior loop does not extend behind the ovary while in large, mature specimens, it may form convolutions between the female complex and the anterior testis. In front of the ovary, the uterus extends anteriorly as a moderately sinuous tube and joins the prominent metraterm with its conspicuous lining of spines. The vitelline follicles are diffuse and confined to the region between the cirrus and the anterior testis. The transverse vitelline ducts, which unite to form the vitelline reservoir, are ventral to the remainder of the female complex.

In large worms, the eggs are fairly numerous, and in fresh material measure from 0.042 to 0.052 mm. long and 0.024 to 0.026 mm. wide. They are fairly thick-shelled and operculate; older ones are brown in color and contain active embryos, but eggs teased from worms and kept for ten days in sea water failed to hatch.

The testes (Fig. 1) lie near the posterior end of the body, one behind the other or slightly overlapping. The vas efferens of the anterior testis is to the left of the median line while that of the posterior testis is to the right. The left vas efferens passes between Laurer's canal and the vitelline duct of that side, while the right runs through the loop of the oviduct. Anterior to the female complex, the vasa efferentia unite to form the vas deferens which is about as long as the seminal vesicle and slightly enlarged posteriorly. The cirrus sac is longer than the metraterm and contains the seminal vesicle, pars prostatica, and prominent, spined cirrus. A short distance posterior to the ventral sucker, the metraterm and cirrus sac join the slender, tubular genital sinus. The largest spines of the metraterm and cirrus (Fig. 2) are about 0.04 mm. long, and of similar structure, but slightly different in shape, those of the metraterm being a little wider. Each spine consists of an acute, refractile point and a very delicate, hyaline medullary and basal portion, and is very different from the body spines in structure, as noted by Olsson (1867) and Ward (1938). In one aberrant specimen, spines similar to those of the metraterm were observed in the genital sinus.

The excretory system (Fig. 11) has been completely traced in young worms obtained from both naturally and experimentally infected eels. The excretory pore is at the posterior end of the body. The vesicle is sac-shaped and extends anteriorly as far as the anterior testis. The main excretory tubules are ciliated throughout their length and extend from the anterolateral edges of the vesicle almost to the ventral sucker where each receives an anterior and a posterior collecting tubule. Each of these tubules serves three groups of flame cells but the disposition of secondary tubules and numbers of flame cells in the groups are not the same for anterior and posterior halves of the system. These differences may be noted in Fig. 11. There may be five, six, or even more flame cells in the posterior-most group on each side, depending on the size of the worm. One flame cell of this group is larger than the others and has a more conspicuous capillary which joins the collecting tubule separately from and anterior to the capillaries of the remaining flame cells of the posterior group. There is also a marked lack of dichotomy in the arrangement of the capillaries in other flame cell groups. The excretory formula of fairly young adults is $2[(3+5+7)+(4+4+6)]=58$ flame cells. There is an increase in the number of flame cells during post-cercarial development but only in the groups associated with the posterior collecting tubules.

The Cercaria (Figs. 7–8)

Specific Diagnosis.—Distome modified trichocercous type. Body pyriform in shape with maximum width anterior to ventral sucker; spi-

nose anterior to eye-spots; extended length over 0.4, contracted less than 0.13. Tail extended 0.33, contracted 0.19. Measurements of 10 cercariae killed by heat: body length 0.21–.28 (average 0.25); maximum width 0.08–.1 (0.086); length of tail 0.2–.23 (0.21), width near base 0.026–.03 (0.028); oral sucker diameter 0.04–.045 (0.043); ventral sucker 0.043–.045 (0.044), situated in posterior half of body; average length of prepharynx 0.023, pharynx 0.017; eye-spots 0.011 wide. Tail typically with a ventral fin-fold and 6 pairs of ventrolateral tubercles, each with a long delicate "hair." Prepharynx moderately long, pharynx present, intestinal ceca extending almost to posterior end of body. At least eight pairs of cephalic glands anterior to ventral sucker; numerous gland-like cells and refractile droplets throughout body. Genital primordium an undifferentiated mass posterior to ventral sucker. Excretory vesicle sac-shaped, thick-walled and with numerous concretions. Main excretory tubules ciliated. Flame cell formula $2[(3+5+7)+(3+3+5)]=52$ flame cells. Develop in rediae in the branchial region and digestive gland of the molluscan host.

Host: Bittium alternatum (Say).

Locality—Waquoit Bay, Falmouth, Massachusetts, U. S. A.

Study of the excretory system of the cercaria in the light of observations on the adult stage necessitates correcting our preliminary abstract (Cable and Hunninen, 1940). The excretory formula of the cercaria, as included in the above specific diagnosis, is not in agreement with that given in the abstract. The flame cells and capillaries of the cercaria are extremely small and the presence of numerous glands and refractile droplets makes it very difficult to observe details of the excretory system.

EXPLANATION OF PLATE II

(All figures concern *Deropristis inflata*)

FIG. 7. Cercaria, ventral aspect.

FIG. 8. Cercaria, median sagittal (optical) section of anterior end showing papilla resembling stylet (free-hand).

FIG. 9. Redia, showing excretory system of one side (free-hand).

FIG. 10. Five-day metacercaria.

FIG. 11. Excretory system of young adult (free-hand).

ABBREVIATIONS

CG, cephalic glands.
CT, caudal tubercle.
EC, excretory concretion.
EP, excretory pore.
EV, excretory vesicle.
EY, eye-spot.
GD, cephalic gland ducts.
IN, intestine.
OS, oral sucker.
PG, genital primordium.
PH, pharynx.
PP, prepharynx.
PS, stylet-like papilla.
RD, refractile droplets.
VF, ventral fin-fold.
VS, ventral sucker.

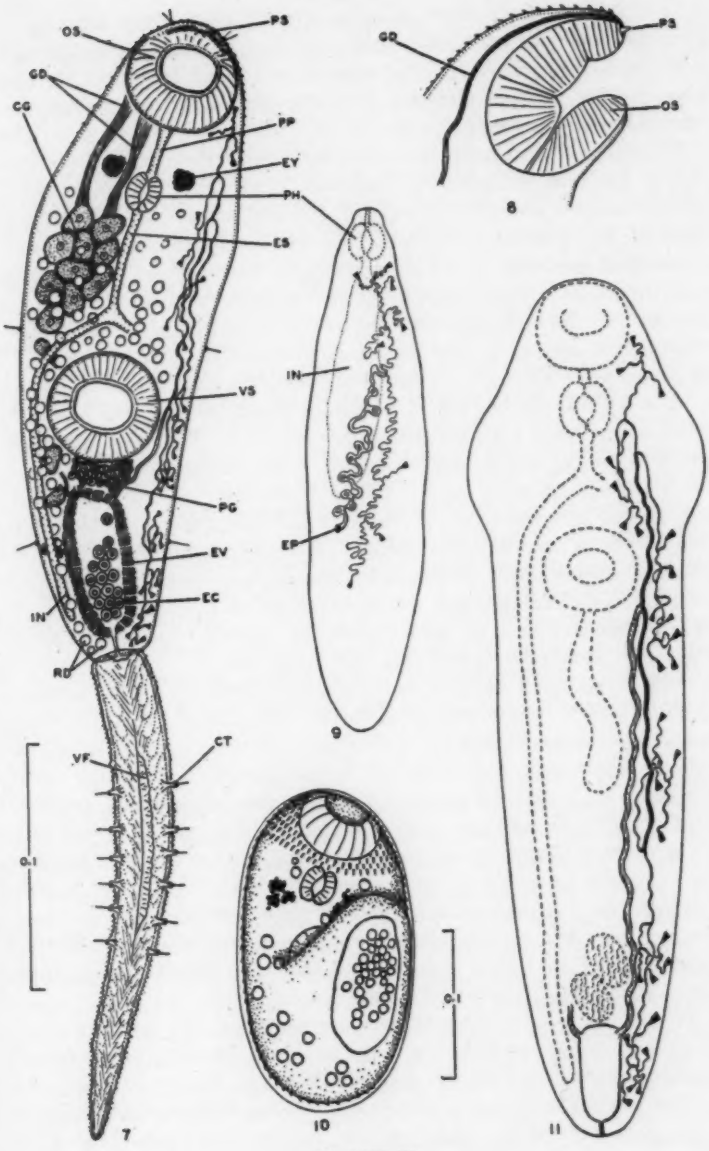
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PLATE II

For several hours after emerging from the snail, the cercariae of *D. inflata* swim almost continuously, tail first, lashing in a figure eight, with the body flexed so that the anterior end is pointed in the direction of swimming. When dissected from the snail, they are photopositive but naturally emerging cercariae are decidedly photonegative and collect at the side of the dish farthest from the light. Precisely the same behavior has been described by Hopkins (1937) for the cercariae of *Anallocreadium* and *Microcreadium*. In one set of experiments with alternate illumination of opposite sides of a syracuse dish, it was observed that when the direction of the light was reversed, the cercariae would swim as a rather compact group across the dish in less than one minute. By illuminating both sides of the dish at once, the larvae scattered, swimming in a hesitant manner. In many cases, decaudation was noted after which the cercarial body remained alive for several hours.

The body of the cercaria is widest apparently at the level where the prominent cervical expansions will develop later. It tapers posteriorly and is devoid of general pigmentation. The eye-spots are conspicuous and contain a refractile body. When contracted the tail is almost as wide as the posterior end of the body and has finely crenated edges. The fin-fold is narrow and crinkled. Although the tail typically has six tubercles symmetrically arranged along each side, there is considerable variation in the number, size, and arrangement of these structures. Accessory tubercles may be present and one specimen was observed in which they were seen on only one side of the tail.

The dorsal lip of the oral sucker bears a blunt, papilla-like structure (Fig. 8) which is difficult to interpret. It is about 0.004 mm. long, conical, non-refractile, and set in what appears to be a slight depression. This structure was first regarded as possibly a rudimentary stylet. However, it is directed anteriorly rather than dorsally as in "allocreadiid" cercariae and has more the appearance of a papilla; the ducts of the cephalic glands do not converge toward it but instead open in a row along the dorsal lip of the oral sucker. We are therefore inclined to believe that the structure in question is not homologous with the stylet of other cercariae. Consequently, we can see no reason to postulate that the cercaria of *D. inflata* is an intermediate type between the ophthalmoxiphidiocercariae and trichocercous forms.

Of the described species of trichocerous larvae, the cercaria of *D. inflata* most closely resembles the cercariae of *Anallocreadium* and *Microcreadium* as described by Hopkins (1937). In fact, it was this resemblance that first led us to suspect that *Homalometron pallidum* might be the adult stage of the larva we were studying. A comparison of the cercariae of *Anallocreadium, Microcreadium, Deropristis,* and *Stepha-*

nostomum will be given in connection with an analysis of the family Acanthocolpidae in the discussion.

The Redia (Fig. 9)

Two redial generations were observed. The simple elongate daughter redia attains a length of over 1 mm. In living specimens, the pharynx measures 0.045 mm. in diameter and is followed by a short, expanded intestine with orange-yellow contents. There is a birth pore near the pharynx. Cercariae reach apparently complete development in the redia which may contain as many as 14 active larvae. In addition to cercariae, even the largest redia contains a group of germ cells situated at the posterior end of the brood cavity.

There is a striking homology between the excretory system (Fig. 9) of the daughter redia and that of the cercaria. Not only are the main excretory tubules ciliated in both stages, but the branching of the tubules and the number of flame cell groups are essentially the same, although with the exception of the anterior-most, the flame cell groups of the cercaria and adult are represented in the redia by single flame cells. As compared with the adult, then, the daughter redia has the complete flame cell complement in only the anterior-most group while the cercaria has the adult complement in three groups or all those connected with the anterior collecting tubule. While we have not had favorable material for determining the excretory patterns of the miracidium and mother redia, the above observations afford convincing evidence of recapitulation in the development of the excretory system in the various life history stages.

The Metacercaria

When annelids were exposed to large numbers of cercariae, massive infections were obtained. The larvae encyst mostly in the axial body region of *Nereis,* although in heavy infections, they occur to a less extent in the parapodia. The cyst is oval in shape, and the primary cyst membrane is thin, and difficult to remove from the worms without injuring them. In older metacercariae, the cysts may become brown in color, particularly when localized in the parapodia. Ten living 5-day metacercariae (Fig. 10) measured 0.17–.23 mm. long and 0.11–.14 mm. wide. The metacercaria does not increase appreciably in size with age although a certain amount of development occurs. Five days after encystment, the sharp-pointed body spines, characteristic of the adult, are present well behind the ventral sucker and those which later become very prominent on the dorsal hump and at the sides of the forebody are just detectably larger than the remaining body spines. Also at this age, the

eye-spots begin to dissociate and the concretions of the excretory vesicle have more the appearance of oil droplets than in the cercaria.

Discussion

The results of the present study and a review of the literature demonstrate certain fundamental differences between *Deropristis* and other genera heretofore allocated to the family Acanthocolpidae. A redefinition of the family will be helpful in summarizing these differences.

Family Acanthocolpidae Lühe, 1909, emend.

Oval or elongate distomes, body weakly muscular, oval to round in cross-section. Cuticle spinose, frequently with large spines associated with oral sucker. Remnants of cercarial eye-spots often present. Suckers relatively close together. Prepharynx usually long, pharynx well developed, esophagus short; intestinal ceca extend almost to posterior end of body, rarely joining the excretory vesicle or with diverticula. Excretory vesicle tubular, not reaching ventral sucker, and with a pair of main excretory tubules extending from its anterior end to cephalic region of body before receiving secondary tubules. Genital pore median or submedian, immediately anterior to ventral sucker or rarely opening within ventral sucker; genital atrium usually tubular, posterior portion sometimes with spines; cirrus sac present, metraterm and cirrus often with prominent spines. Testes two (rarely multiple), in posterior region of body, tandem or obliquely one in front of the other; ovary near or some distance in front of anterior testis; seminal receptacle lacking, uterine seminal receptacle frequently present; vitelline follicles numerous, reaching from level of cirrus sac to posterior end of body. Uterine coils preovarian and intercecal, not extensive; eggs relatively few, operculate and without filaments. Adults parasitic in intestine of marine fishes, metacercariae in fishes. Cercaria of ophthalmoxiphidiocercous type, developing in simple rediae in marine gastropods. Type genus, *Acanthocolpus* Lühe, 1906. Includes also:

Stephanostomum Looss, 1899 (syn. *Stephanochasmus* Looss, 1900; *Lechradena* Linton, 1910).
Dihemistephanus Looss, 1901.
Neophasis Stafford, 1904 (syn. *Acanthopsolus* Odhner, 1905).
Tormopsolus Poche, 1925.
Echinostephanus Yamaguti, 1934.
Lepidauchen Nicoll, 1913.
Pleorchis Railliet, 1896 (syn. *Polyorchis* Stossich, 1888, preoccupied).
Pseudolepidapedon Yamaguti, 1938.

A careful comparison of the above genera indicates a sufficiently close interrelationship to preclude the necessity of subfamilies.

In contrast with the family Acanthocolpidae as set forth above, *Deropristis* has a modified trichocercous cercaria which encysts in annelids; the main excretory tubules barely reach the ventral sucker; there is a well developed seminal receptacle independent of Laurer's canal; vitelline follicles are scanty and restricted to the region between the cirrus sac and the anterior testis; the uterine coils extend into the space between the ovary and anterior testis; and the prepharynx is shorter than the esophagus. These differences fully justify the exclusion of the genus *Deropristis* from the Acanthocolpidae.

The above definition of the family excludes also the species described by Little (1930) as *Dihemistephanus sturionis;* it is very doubtful whether this form belongs in either the genus *Dihemistephanus* or the family Acanthocolpidae, since Laurer's canal is enlarged to form a seminal receptacle, the vitelline follicles as figured appear scanty and do not extend to the extreme posterior end of the body while the uterus does, and the eggs are small and numerous. Also, the cephalic spines apparently are not as closely associated with the oral sucker as in *D. lydiae* (Looss, 1901; Nicoll, 1909), being arranged more like those of the echinostome collar and lacking the two rows of smaller spines in the ventral gap.

Dihemistephanus brachyderus Manter, 1940, is in agreement with the family diagnosis in most respects although it has two ascending excretory tubules on each side formed by the division of each main tubule a short distance from the vesicle. We have examined a specimen of *D. brachyderus,* kindly provided by Professor Manter, and found that the female complex is typical of the Acanthocolpidae; a Laurer's canal is present and the beginning portion of the uterus is expanded to form a prominent receptaculum uterinum, a true seminal receptacle being absent. In all other respects except the ascending excretory tubules, which may represent an aberrant condition, *D. brachyderus* is typically acanthocolpid in structure, although its allocation to the genus Dihemistephanus is somewhat uncertain, as stated by Manter (1940). On the whole, however, *D. brachyderus* resembles *D. lydiae,* the type species, far more than does *D. sturionis.*

The descriptions of *Lepidauchen hysterospinosa* Manter, 1931, and *Pleorchis sciaenae* Yamaguti, 1938, extend our knowledge of genera hitherto imperfectly understood, so that they now may be included in the family Acanthocolpidae. Also, *Pseudolepidapedon* is unquestionably an acanthocolpid. *Lepidauchen* and *Pseudolepidapedon* differ from the

original genera of the family chiefly in respect to the terminal genitalia, a character whose taxonomic significance is often unreliable and has been greatly overestimated in proposing supergeneric groups. The presence of intestinal diverticula and multiple testes in *Pleorchis* are of not more than generic significance (compare *Fasciola* and *Fasciolopsis; Siphodera* and *Exorchis*). Since the genera *Pleorchis* and *Schistorchis*, for which Poche (1925) erected the family Pleorchiidae, do not seem to be at all closely related, this family must be judged invalid.

Although the acanthocolpids bear certain resemblances to the echinostomes, as noted by many authors, life history studies indicate that their closest relatives are to be sought among the allocreadiid trematodes having ophthalmoxiphidiocercariae. These include *Allocreadium, Crepidostomum, Megalogonia, Bunodera* and related genera. The family Allocreadiidae [2] should be restricted to include these forms. However, the acanthocolpids differ from the allocreadiids in morphology and host-parasite relationships. As far as known at present, allocreadiid cercariae develop in freshwater bivalves and encyst in invertebrates while acanthocolpid cercariae develop in marine gastropods and encyst in fishes. The chief morphological difference is in respect to the excretory system. In view of these differences, the Acanthocolpidae and Allocreadiidae are regarded for the present as distinct families of a common superfamily.

The systematic position of the genus *Deropristis* remains to be discussed. There seems no alternative to the conclusion that its nearest relatives are such forms as *Homalometron, Anallocreadium, Microcreadium* and *Lepocreadium*. In our opinion, the subfamilies Lepocreadiinae and Anallocreadiinae and the genus *Deropristis* constitute a family independent of the Allocreadiidae. As far as we have been able to determine, the only published proposal of such a combination is the grouping of several genera under the heading, Lepocreadiidae, in the Zoological Record for 1934. Since this list presumably is attributable to Nicoll, the sectional editor, the family is defined below under the following heading:

[2] The authors' (Hunninen and Cable, 1941) recent proposal to restrict the family Allocreadiidae to contain those trematodes having in common cotylomicrocerous cercariae and certain other characteristics was based on the assumption that *A. angusticolle* was actually a species of *Allocreadium* since it did not violate the original description of the genus. The description of *A. isoporum*, the type species, was unavailable at the time our paper was revised. This description fully justifies Dobrovolny in transferring *A. angusticolle* to *Plagioporus;* although Looss performed no life history experiments with the ophthalmoxiphidiocercaria which he believed to be the larva of *A. isoporum*, it is very likely that his belief was correct. We therefore retract our proposal in favor of that of Hopkins (1941), viz., that the name Opecoelidae be used for the restricted family.

Lepocreadiidae Nicoll, 1934

Medium sized distomes, body variable in shape, rarely without spines. Mouth subterminal, prepharynx, pharynx, and esophagus present; intestinal ceca almost always long, rarely joining excretory vesicle or with separate anal openings. Excretory vesicle tubular, variable in length, frequently reaching pharyngeal level; rarely Y-shaped and then with short arms not receiving main tubules; excretory pore posterior, terminal or subterminal. Main excretory tubules never reach cephalic region but divide near acetabular level to form anterior and posterior collecting tubules. Flame cells numerous. Genital pore anterior to ventral sucker, median or displaced to left; rarely marginal or with a "genital sucker." Cirrus sac present or absent; if present almost always followed by an external seminal vesicle. Testes in posterior half of body, occasionally multiple, usually two, median and tandem, sometimes diagonal or opposite in short bodied forms. Ovary pretesticular, rarely behind level of anterior testis. True seminal receptacle and Laurer's canal present. Vitellaria usually extensive, often filling body posterior to ventral sucker, rarely confined between levels of cirrus sac and anterior testis or restricted to post-testicular region of body. Uterus preovarian in almost all genera and not extensive; eggs relatively few and large. Parasites of fishes. Cercariae of trichocercous type, stylet lacking, with conspicuous eye-spots remnants of which may persist in adults. Develop in simple rediae in gastropods, encyst in invertebrates. Includes the following subfamilies:

Lepocreadiinae Odhner, 1905

With characters of the family. Genital pore to left of median line; cirrus sac present, followed by external seminal vesicle which is sometimes embedded in a glandular mass. Vitellaria well developed, reaching posterior end of body; uterus preovarian in almost all genera. Parasites of marine fishes. Type genus *Lepocreadium* Stossich, 1903. Includes also:

Lepidapedon Stafford, 1904 (syn. *Lepodora* Odhner, 1905).
Opechona Looss, 1907 (syn. *Pharyngora* Lebour, 1908).
Aephnidiogenes Nicoll, 1915.
Pseudocreadium Layman, 1930 (syn. *Leptocreadium* Ozaki, 1936).
Diploporus Ozaki, 1928 (syn. *Bianium* Stunkard, 1930).
Multitestis Manter, 1931.
Rhagorchis Manter, 1931.
Lepotrema Ozaki, 1932.
Myzoxenus Manter, 1934.

Lepocreadioides Yamaguti, 1936.
Hypocreadium Ozaki, 1936.
Labrifer Yamaguti, 1940.
Allolepidapedon Yamaguti, 1940.
Opechonoides Yamaguti, 1940.

Homalometrinae nom. nov. pro Anallocreadiinae Hunter and Bangham, 1932

With the characters of the family. Body not very elongate, with or without spines. Genital pore median, immediately in front of or rarely posterior to ventral sucker; cirrus sac lacking. Vitellaria well developed, rarely restricted to post-testicular region. Parasites of freshwater and marine fishes. Type genus *Homalometron* Stafford, 1904 (syn. *Anallocreadium* Simer, 1929). Includes also:

Microcreadium Simer, 1929.
Crassicutis Manter, 1936.
Myzotus Manter, 1940.
An unnamed genus reported by Manter (1941).

Deropristiinae subf. nov.

With characters of the family. Body elongate and spinose with enlarged spines on the middorsal and lateral regions of forebody. Genital pore median, anterior to ventral sucker; cirrus sac present. Cirrus and metraterm with prominent spines; genital sinus tubular. Ovary and anterior testis separated by a space into which the uterus may extend. Vitellaria scanty, between levels of cirrus sac and anterior testis. Parasites of migratory fishes; larval stages marine. Type and only genus *Deropristis* Odhner, 1905.

Since the subfamilies Lepocreadiinae and Homalometrinae differ chiefly in respect to the terminal genitalia, their validity may be questionable. However, differences in their cercariae and the fact that both subfamilies have become established in the literature probably justify their retention. Manter (1926) expressed the view that the genera *Anallocreadium* and *Homalometron* probably are synonymous. We can find no basis for maintaining the separate identity of these genera. Since *Homalometron* has clear priority, the name *Anallocreadium* is not available for the subfamily Anallocreadiinae proposed by Hunter and Bangham (1932). In restudying Stafford's collection, Miller (1941) has expressed the opinion that *Homalometron* and *Lepocreadium* may be synonymous. We have placed these genera in separate subfamilies and fail to see the reason for Miller's opinion since *Lepocreadium* has a long

excretory vesicle and cirrus sac while *Homalometron* has a short vesicle and lacks a cirrus sac. Incidental to the present study, we have determined that the excretory system of *H. pallidum* is very similar to that of *Deropristis,* the chief difference being a much larger number of flame cells in *Homalometron,* particularly in the anterior groups.

The trematodes assigned by various authors to the families Gyliauchenidae and Opistholebetidae present a perplexing problem which Manter (1940) has discussed recently. Travassos (1934) and Ozaki (1937) have assigned these families to the superfamily Paramphistomoidea, although they contain genera ranging from the usual distome type to forms superficially resembling the amphistomes. Figuring largely in this disposition was the presence of lymphatic vessels in certain genera. Manter (1940) pointed out the resemblance of certain gyliauchenids to the Lepocreadiinae and Anallocreadiinae and described what appeared to be lymph channels in species of *Opechona* and *Pseulepidapedon.* On the other hand, he cites Carassotrema, a gyliauchenid, which lacks lymphatic vessels, indicating that their presence does not necessarily denote amphistome affinities. Manter therefore concluded that the family Gyliauchenidae probably should be considered in the superfamily Allocreadioidea.

A study of Ozaki's (1937) excellent descriptions of the gyliauchenids affords convincing evidence that they are aberrant forms closely related to the family Lepocreadiidae. Their peculiar body shape can be explained by failure of the hindbody to grow during post-cercarial development, with a compensating elongation of the forebody and a resultant shifting of certain internal organs anteriorly. The so-called excretory papilla then would correspond to the post-acetabular region of ordinary distomes. Several morphological features support this explanation. First, the genera *Petalocotyle, Telotrema, Flagellotrema* and *Gyliauchen* comprise a fairly complete series of body forms ranging from the usual distome type to one resembling the amphistomes. In this series, there is a progressive elongation of the prepharynx and decrease in the length of the intestinal ceca until they are little longer than the pharynx in *Gyliauchen.* There is also a progressive decrease in the length of the excretory vesicle and retreat of the testes from the hindbody which becomes too small to contain them. Elongation of the forebody is suggested not only by the length of the prepharynx, but also by the distance separating flame cell groups near the middle of the body; the genital pore and ventral sucker become widely separated. Furthermore, the lymphatic vessels which extend posteriorly only to the ventral sucker or about one-fourth the body length in *Petalocotyle,* likewise reach the ventral sucker in *Telotrema* but are consequently almost as long as the body in

this species. Progressive reduction of the hindbody is indicated not only by the shortening of the intestinal ceca and excretory vesicle, but also by the manner in which the flame cell groups are crowded in the hindbody of the intermediate form, *Telotrema*, and apparently become reduced in number in *Flagellotrema*.

The genital complex and excretory system of the gyliauchenids agree well with those of the lepocreadiids and certainly are unlike those of typical amphistomes. In no case, is the ovary posterior to the testes as in most amphistomes and only in *Flagellotrema*, where it lies between the testes, does it approach the condition found in certain short-bodied amphistomes. The reduction and anterior displacement of the vitellaria may be correlated with the shortening of the intestinal ceca and compensating elongation of the prepharynx. The somewhat shortened ceca and arrangement of the reproductive system in the lepocreadiid *Opechonoides* are suggestive of certain gyliauchenids.

On the basis of structure, it could be argued that either the amphistome or the distome is the primitive gyliauchenid body type. This question cannot be answered until the larval stages are discovered. In view of the fundamental morphological resemblances of the gyliauchenids to the lepocreadiids, we believe that the family Gyliauchenidae does not belong in the Paramphistomoidea but instead in a common superfamily with the Lepocreadiidae. Further study may in fact necessitate the combination of these families if it is shown, as some observations indicate, that the lymphatic system in trematodes is correlated with physiological requirements that may arise independently in various groups.

Secondary acquisition of amphistome characteristics in the family Opistholebetidae is suggested by the series *Maculifer, Heterolebes,* and *Opistholebes*. There is some suggestion that this group may be related to the Opecoelidae but the evidence is not sufficiently clear to postulate affinities. This is true also of the family Cephaloporidae.

It is uncertain whether the families Allocreadiidae (*sensu stricto*), Opecoelidae, Lepocreadiidae, Acanthocolpidae, and Gyliauchenidae represent only one or more than one superfamily. Our knowledge of life histories and embryological development, particularly of marine forms, is still fragmentary and it is possible that in certain groups the few life histories known are of species having atypical larval stages such as have been described in other groups and would by themselves be misleading as a basis for judging relationships.

Summary

The life history of *Deropristis inflata* has been demonstrated experimentally. The cercaria is a trichocercous form developing in simple

rediae in the marine snail, *Bittium alternatum*. It encysts in *Nereis virens* and the eel, *Anguilla rostrata* serves as the natural definitive host which becomes infected by eating annelids containing metacercariae.

The genus *Deropristis* and *Dihemistephanus sturionis* are excluded from the family Acanthocolpidae which is redefined to include for the first time the genera *Lepidauchen, Pleorchis,* and *Pseudolepidapedon*. The family Pleorchiidae thereby becomes invalid.

It is proposed that the family Allocreadiidae be restricted to include only forms known or believed likely to have ophthalmoxiphidiocercariae with main excretory tubules not reaching the cephalic region of the body before receiving secondary tubules. The Allocreadiidae and Acanthocolpidae are regarded as separate but closely related families.

The genus *Anallocreadium* is reduced to synonymy with *Homalometron* and the subfamily Anallocreadiinae is renamed Homalometrinae. The Lepocreadiinae, Homalometrinae, and new subfamily Deropristiinae are regarded as a distinct family, the Lepocreadiidae. The family Gyliauchenidae is believed to be nearer the Lepocreadiidae than the Paramphistomoidea. Whether the Allocreadiidae, Acanthocolpidae, Opecoelidae, Lepocreadiidae and Gyliauchenidae constitute only one or more than one superfamily is not apparent from the fragmentary knowledge of life histories and embryology.

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STUDIES OF THE RESPIRATORY METABOLISM OF WARM AND COOL SPRING FISHES [1]

F. B. SUMNER AND URLESS N. LANHAM

I. Introduction

It is generally agreed that the respiratory metabolism (oxygen consumption) of poikilothermal animals tends to vary directly with the temperature to which they are subjected. Less familiar is the existence of a process of thermal adaptation, following transfer from one temperature to another. The immediate effects of such transfer may be succeeded by a "rebound" from the extreme condition first induced back toward the previous condition of the animal. A further illustration of the same principle is the fact that the metabolic rate of fishes at any given temperature may be considerably influenced by their previous temperature experience. Fishes which had been acclimatized to 30°, for example, showed a lower rate of oxygen consumption, when transferred to 20°, than ones which had been kept continuously at 20°, and conversely, transfer from 10° to 20° resulted in a higher rate than that shown by fishes accustomed to the latter temperature. Here, too, any such departure from the normal oxygen consumption for a given temperature is followed, within a few days, by a process of adaptation which tends to equalize the metabolic rate, regardless of previous history (Wells, 1935*b*; Sumner and Wells, 1935; Sumner and Doudoroff, 1938).

An obvious question is whether the metabolic rate of fishes and other poikilothermal animals in nature varies as widely with the temperature of their medium as might be inferred from these laboratory results. Or, may not more far-reaching processes of adaptive modification occur in nature, such that warm-water and cold-water derivatives of the same stock come to have nearly the same rates of metabolism?

Fox and Wingfield [2] compared certain closely related species, or local representatives of the same species, living at different latitudes, and reported varying degrees of thermal adaptation of this sort. In some cases, there was no such adaptation, the two forms under comparison differing widely under their own normal conditions of life, but approximating one

[1] Contributions from the Scripps Institution of Oceanography of the University of California, La Jolla, California, New Series No. 157. Services were rendered in the course of these studies by persons working under W.P.A. Project 65–1–07–2317.

[2] Fox, 1936, 1939*a*, 1939*b*; Fox and Wingfield, 1937; Wingfield, 1939.

another closely when their rates of physiological activity were compared at equal temperatures. In other cases, the rates of these functions were nearly identical in their respective habitats, despite considerable differences of temperature. Here extensive adaptation appears to have taken place.

In 1939 Sumner and Sargent made field studies of the respiratory metabolism of fishes in springs of varying temperature in Nevada. Experiments were conducted upon warm and cool spring representatives of species belonging to two genera, *Cyprinodon* and *Crenichthys* (Sumner and Sargent, 1940). In these studies, the rapidity of death in thousandth-normal KCN was used as an index of the rate of respiratory metabolism, the two, as is well known, being directly correlated.

In both of these genera, the warm-spring fishes, at their accustomed temperature, succumbed far more rapidly than the cool-spring fishes at their accustomed temperature. As regards the behavior of warm-spring individuals, transferred to the cool spring, the two fishes seemed to give different results, though the data hardly warrant definite conclusions on this point. It should be pointed out, however, that the seeming lack of adaptive modification of the metabolic rate on the part of *Crenichthys* in warm water has been borne out by the studies to be reported herewith. Its striking resistance to the lethal effects of high temperature is something quite distinct (Sumner and Doudoroff, 1938).

The importance of the physiological and ecological problems here involved, and the widespread human threat to the continued availability of warm springs to naturalists (Brues, 1928) seemed to warrant a second trip to the springs previously visited in our studies of *Crenichthys*. This work was carried on during portions of September and October, 1941.

While we believe that the publication of our present results calls for no apologies, it should be pointed out that field studies of this sort should not be judged by the same standards as are applied to similar studies in the laboratory. This is because of the impossibility of transporting important articles of laboratory equipment, unfavorable weather conditions, and limited time (in this case, less than 4 weeks) at the scene of our operations.

We must here acknowledge valuable help received at various times from our colleagues, Dr. Denis L. Fox and Dr. Marston C. Sargent, and from our companion in camp, Mrs. Margaret Sumner.

II. Methods

Altogether, 200 fishes were employed in these studies, while more than 200 oxygen determinations were made. The fishes were readily caught

at both of these springs with hand-nets. Some time (several hours to a day or more) before the respiration tests were made, small lots (6 to 9) of the fishes, of roughly uniform size, were placed in each of four glass tubes having a length of 40 cm. and an internal diameter of 4 cm. Except during the period of a test, wide-mesh cloth screen was fastened over the flanged ends of the tubes, which were laid, side by side, in a screen cage, placed at the bottom of the stream, and directly in the line of the current. When a test was to be made the screens were removed from one of the tubes, and close-fitting rubber stoppers, one of these carrying a glass stopcock, were forced into its ends. The tube was then returned to its former position in the cage. The capacity of these tubes, with the stoppers in place, ranged from 439 to 454 ml.

At the close of 15 minutes (10 in some experiments) the water-samples were drawn off into glass-stoppered bottles of about 150 ml. capacity. A glass tube, continuous with the stopcock, was inserted into the bottle, down to its bottom, and the water allowed to overflow from the bottle's mouth. Two samples were invariably drawn in each test.[3] Time records, in seconds, were kept of each of these steps with a stop-watch. Within a few minutes of the drawing of each sample, the Winkler reagents were introduced into the bottles. Titration was performed on either the same or the next day.[4]

In recent studies of oxygen consumption in fishes (cf. Keys, 1931; Wells, 1935*a*; Wunder, 1936; Leiner, 1938) it has been the more usual practice to hold the animals in a continuous current of water, and to draw samples from this at intervals, rather than to hold them, tightly stoppered, in an inclosed volume of water. Under the former conditions, the accumulation of carbon dioxide and other possible metabolites is, of course, prevented. Likewise the fishes are subjected to less disturbance in the taking of water samples. It is obvious, however, that such procedure would be difficult, if not impossible, to employ in the field. Moreover, the highest of our CO_2 concentrations probably fell well short of two thousandths of an atmosphere. No signs of discomfort were visible at the close of the 15-minute period, at least in the cooler water. So far as disturbance is concerned, the fishes showed little evidence of excitement except at the beginning and the end of the test. Most of the time they swam quietly or remained at rest. In this connection, it is worth pointing

[3] The values for oxygen consumption employed in our tables and graphs have been based entirely upon the first of these samples. The second figures have been regarded as less representative for a number of reasons.

[4] No appreciable effect of delaying the titration over night is probable. Readings of four divided samples, titrated on the same and on the following day, gave mean values of 2.48 and 2.52 respectively. These were as close as the average divided samples titrated on the same day.

out that our figures show no more variability for fishes of a given size than do those of Wells (1935*a*, pp. 209–210) in experiments with *Fundulus*, where a running-water system was employed. Indeed Wells has recorded fully as great differences as ours when repeating tests of *the same lots* of fishes.

Before and after the performance of a series of these tests, and frequently alternating with them, water samples were withdrawn from the spring, as near as possible to where the tubes had lain.

Owing to the possible presence of nitrites in our samples (cf. Allee and Oesting, 1934) the modified Winkler method (A.P.H.A., 1936) [5] was used in all cases, with a few exceptions which were performed for the sake of comparison.[6]

Under field conditions, it is hardly to be expected that such high precision will be attained as is possible in the laboratory. Thirty cases in which duplicate water samples were drawn from a single tube revealed a mean difference of 2.2 per cent between them. Lack of homogeneity ("streakiness"), in the water, to be discussed presently, may have had its effect, even within our half-liter tubes.

At the close of each test of oxygen consumption, the fishes involved were dried upon paper towels and weighed. The aggregate weight of each lot, together with the estimated original oxygen titre of the water, the oxygen titre at the close of the test, the volume of water in the tube (allowing for volume occupied by the fishes), and the exact duration of the test, are the data upon which the metabolic rates (ml./gm./hr.) are based.

Unfortunately, the equipment at our disposal did not permit of our collecting a sample of the water admitted to the tube at the moment of commencing each test of oxygen consumption. For this reason, the previous oxygen titre of the water which was actually "breathed" by the fishes in any given experiment cannot be stated with precision. As will appear later, water samples from these springs, taken at different times or in slightly different positions, might differ considerably in their oxygen titre. While this circumstance introduces a considerable margin of possible error into the result of any single titration, we believe that the validity of our chief comparisons is little affected. This (1) because the

[5] Our procedure differed from the procedure therein stated in that in the succeeding steps of the test (Winkler proper) HCl was used instead of H_2SO_4, while manganous chloride was used in place of the sulphate. This is in accordance with the procedure long employed at the Scripps Institution in analysis of sea-water.

[6] It is doubtful whether the modified procedure was necessary, in the case of the water-samples taken directly from the springs. Four divided samples, of which half of each was subjected to the modified and half to the unmodified Winkler procedure, gave mean values of 2.54 and 2.55 respectively.

differences involved in comparing two series of differing history are great in proportion to errors due to uncertain original oxygen content of the water; and (2) because results based upon two different procedures which we have employed in selecting a figure to represent this oxygen content show substantially the same relations.

The most obvious of these procedures (1) was to select for each computation the oxygen value of that water sample which was taken most nearly at the same time as the performance of the metabolism test under consideration. However, there were instances in which no water sample was taken within an hour or more of this test.

The alternative procedure (2) was to employ, as our basis of calculation, the mean titre of all the water samples taken in the position in which the test in question was made.[7] It is obvious that many single values for oxygen consumption obtained in this way would be no more than rough approximations. It is equally plain, however, that the *average* of a series of values thus obtained could not depart far from an average such as we should obtain if the actual oxygen titres of the corresponding water samples were available.

It is gratifying that graphs based upon these two procedures agree in most essential features, though differing considerably in detail (Figs. 2–4). In the text, the mean of the two resulting values will be given.

A further difficulty in postulating any initial oxygen content for the water is the fact that a quite appreciable amount of oxygen was taken out of their medium by the fishes before the commencement of the test, despite the continuous flow of water through the tubes. This current was undoubtedly retarded somewhere by the screens, which it was necessary to fasten over the ends. Whatever the exact extent of this preliminary depletion of the oxygen, we may probably fairly assume that it did not differ very widely from one experiment to another.

III. The Springs

"Preston Spring," the largest of several springs in the vicinity of Preston, Nevada, is really a group of small springs, discharging near together and giving rise to a creek of considerable size. The water in and near the orifices appears to maintain a constant temperature very close to 21° C.

The high variability of the oxygen values for all of the water samples taken at Preston Spring is indicated by Fig. 1. Reference has already been made to the "streakiness" of this water in respect to oxygen content.

[7] Excluding those taken on cloudy days or near sunset, which regularly gave very low values.

While the concentration at the sources appears to be fairly constant (cf. Sumner and Sargent, 1940), it is considerably lower than that in the rest of the stream, even in their immediate neighborhood. Surface aeration and photosynthesis are, of course, responsible for the differences. The mean of two samples, drawn from what appeared to be the largest orifice was 1.96 ml. per litre, while samples taken a few yards away averaged about 2.60 ml. The resulting variegation in the oxygen concentration of these waters was shown by determinations of samples taken in various positions.

It would be quite erroneous, however, to infer that the water with which we filled our tubes for the tests of respiratory metabolism was

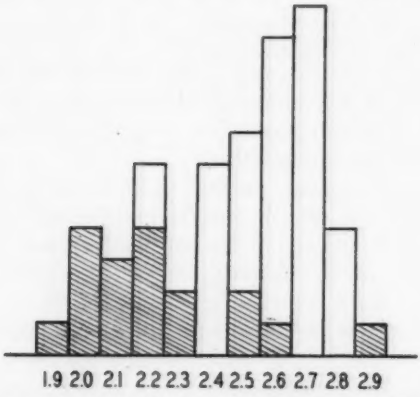

FIG. 1. Oxygen values (ml./l.) for water of Preston Spring. Shaded squares denote samples taken in the first position of our cage directly over one of the minor orifices of the spring; the others in a second position some distance from this.

subject to such an extreme range of variation as is here indicated. Care was taken, moreover, to collect our samples from points close to those where the tests were made.

Some thirty miles to the south, in the same valley,[8] is the warm spring here discussed, known locally as "Mormon Spring." This is a spring of much smaller volume than that at Preston. Its temperature is almost exactly 37° at its source, but the water cools off by a degree or two within a hundred feet downstream. Most of the fishes were caught in water ranging from 35° to 36.5° C., and temperatures such as these prevailed where the experiments were performed. At the lowest point in the stream where fishes were seen, the thermometer recorded 34.8°. Within

[8] Called the "White River Valley," though there is no continuous stream here.

a few hundred feet from its source, the stream spreads out into a marsh, where the water probably follows the temperature of the air. No fishes were found there. It seems probable, therefore, that the Mormon Spring representatives of *Crenichthys baileyi* seldom experience temperatures lower than 34°. On the other hand, individuals were rather frequently seen close to the main source, where the temperature was nearly or quite 37°.

As regards oxygen content, water samples taken well below the surface, in the main orifice of the spring, gave a mean value of 0.51 ml./l., while at a nearby point, where fishes were often seen, this value was 0.65. Samples taken downstream in the creek, where most of the fishes were caught, ranged from 1.54 to 1.88.[9]

IV. The Fishes

Crenichthys baileyi (Gilbert) [10] is the commonest species of fish in Preston Spring, at least near the sources. Besides *Crenichthys*, there are, however, at least three other fishes present. Two of these (*Apocope osculus* and *Lepidomeda sp.*) are Cyprinidae, the other (*Pantosteus sp.*) being a Catostomid. At Mormon Spring, *Crenichthys* is the only fish present. Likewise, it is much less abundant than in Preston Spring. Dr. Hubbs informs us that he regards the two populations of *Crenichthys* here considered as subspecifically distinct. He states (cf. Hubbs and Kuhne, 1937, referring to similar differences in *Apocope*) that the warm spring form has a slightly lower average number of fin-rays than the cool spring form, and counts which we have made of the dorsal and anal rays of about fifty fishes of each race confirm this statement.

Identification of the sexes is difficult or impossible in the smaller specimens mostly used in these studies. Since such individuals were probably sexually immature, it is not likely that sexual differences occurred in their metabolic rate.

V. Oxygen Consumption

Experiments were performed in which each stock was tested in its own waters, and in which each was tested after transfer to the habitat of the other.

Preston at Preston

When all of these fishes are thrown together as a single population (Fig. 2 and Table I), the values for respiratory metabolism (ml./gm.-

[9] Readings for these were as follows: 1.54, 1.56, 1.62, 1.76, 1.78, 1.86, 1.86, 1.88.
[10] Cf. Hubbs, 1932; Hubbs and Miller, 1941. We are indebted to Dr. Hubbs for the identification of the other species.

/hr.) are seen to vary very widely, ranging from 0.123 to 0.390, using in each case the mean of the values obtained by our two procedures (see p. 317). This variation is in part due to the heterogeneous nature of the material. The series includes fishes differing widely in size, this ranging from 0.51 gm. (mean of one lot of 9 individuals) to 9.5 gms. (weight of one large individual tested alone). While the (inverse) correlation between size and metabolic rate is not very close, Figs. 2 and 3 show that

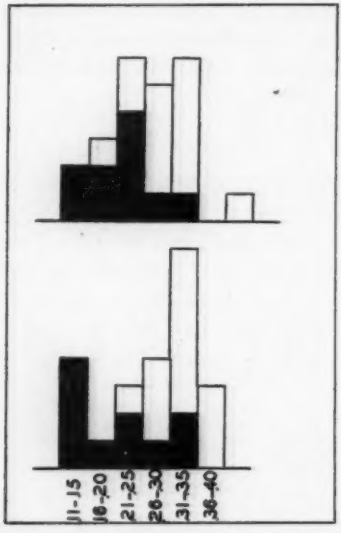

FIG. 2. Rate of oxygen consumption (ml./gm./hr.) of Preston Spring fishes in Preston Spring. Upper histogram is based upon computations (1) in which the O_2 value of the water sample taken at most nearly the same time as the test was used in each case; lower histogram upon computations (2) in which the mean of the entire series of O_2 values (with certain exceptions) was used (see p. 317). Shaded squares denote rate for fishes larger than 1 gram in mean weight, others for fishes less than 1 gram.

such correlation exists. It will be found, too, that the mean of ten values for fishes averaging over 1 gm. in weight is 0.215, that of the thirteen values for fishes below this mean weight being 0.307 (see Fig. 2).

In two cases, special experiments were made with Preston-at-Preston lots, in order that more strict comparisons with the Mormon-at-Mormon series might be possible. Thus, in the case of four lots (designated by asterisks) the period of the test was abbreviated to ten minutes (see be-

low) instead of fifteen minutes as was the rule in the Preston Spring fishes. Again, four other lots (designated by daggers) were tested only a few hours (1½ to 4) after being confined in the tubes, instead of being previously kept through one night, as was commonly done. While the results of both of these tests indicated considerable differences from the general average (13 to 18 per cent), part of this difference appears to

TABLE I

Oxygen Consumption of Fishes (First Sample Drawn)—Preston Spring

(Arranged in order of mean weight)

Lot of fishes	Weight of sample	Number of fishes	Mean weight	ml./gm./hr.				
				Procedure 1		Procedure 2		Means
A	4.6	9	0.51	0.370		0.424		
B	5.1	9	0.57	0.341	0.337	0.307	0.320	0.328
C	5.1	9	0.57	0.278		0.200		
D†	3.5	6	0.58	0.358		0.348		
E†	3.8	6	0.63	0.357		0.271		0.314
F	6.6	9	0.73	0.253		0.293		0.273
G	5.1	6	0.85	0.283	0.275	0.212	0.252	0.264
G'*	5.1	6	0.85	0.268		0.293		
H†	5.4	6	0.90	0.309		0.303		
I	5.4	6	0.90	0.306		0.236		
I'*	5.4	6	0.90	0.333	0.322	0.343	0.306	0.314
J	5.5	6	0.92	0.326		0.317		
J'*	5.5	6	0.92	0.334		0.329		
K	7.0	6	1.16	0.153	0.248	0.222	0.230	0.239
L†	7.0	6	1.17	0.343		0.239		
M	7.8	6	1.30	0.277		0.270		
M'*	7.8	6	1.30	0.311	0.244	0.308	0.261	0.252
N	8.0	6	1.33	0.144		0.204		
O	8.7	6	1.45	0.236		0.222		0.229
P	10.0	6	1.67	0.209		0.227		0.218
Q	11.2	6	1.87	0.179		0.205		0.192
R	5.4	1	5.40	0.150		0.111		0.130
S	9.5	1	9.50	0.136		0.153		0.144
	Means		1.56	0.272		0.262		0.267

have been due to the size of the fishes employed. In any case the chief relations here considered are little affected.

(2) *Mormon at Mormon*

It was difficult for practical reasons (distance and time) to leave the Mormon Spring fishes over night in tubes before making the tests of oxygen consumption in their own habitat, as was customary in the

Preston-at-Preston series. Likewise it was found that the usual fifteen minutes after stoppering was too long a period of confinement, considering the high temperature (36° ±) of the water and its low oxygen content.

Two preliminary lots, however, were subjected to this longer test. The values for oxygen consumption for these lots (mean weights 0.76 and 0.67 gm.) were 0.386 and 0.382, ml./gm./hr., respectively. Some of these fishes showed marked signs of asphyxiation during the last few minutes. That respiratory values for partially asphyxiated fishes cannot be regarded as normal is obvious.

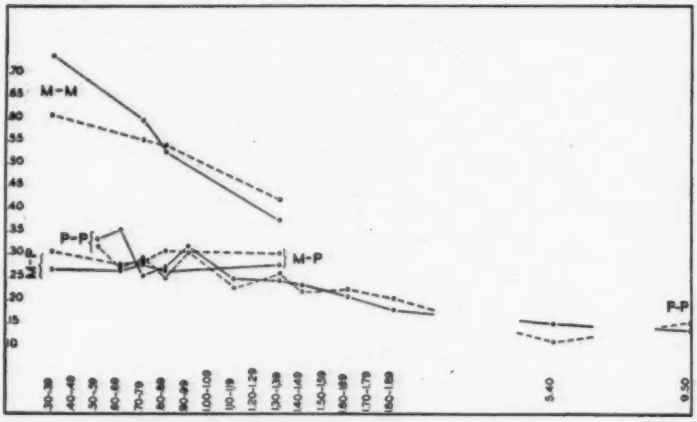

FIG. 3. Comparison of "Preston-at-Preston" series with "Mormon-at-Mormon" and with "Mormon-at-Preston" (1 day). Abscissas = mean weight of fishes in each lot; ordinates = oxygen consumption (ml./gm./hr.). Solid lines connect values obtained by second of above procedures (see legend for Fig. 2); broken lines connect values obtained by the first procedure.

The remaining four tests, limited to ten minutes, are very instructive. In Table II (upper section) it is seen (1) that values for oxygen consumption are far higher in this series than in the "Preston at Preston" series; (2) that these values are inversely correlated with the mean weights of the fishes involved. This correlation is far more evident than in the Preston series, a significant fact which will be discussed shortly. Comparisons between the Mormon-at-Mormon and Preston-at-Preston series may be made by inspection of Fig. 3. That the relations shown here are little affected when we substitute certain more strictly comparable Preston-at-Preston lots, has already been pointed out.

(3) *Preston at Mormon*

It had already been reported by Sumner and Sargent (1940) that Preston Spring fishes speedily die when transferred to Mormon Spring. In the more recent experiments, it was found that the fishes were overcome so rapidly by the heat that no instructive determinations of oxygen consumption were possible.

(4) *Mormon at Preston*

In these experiments, the fishes were transferred from Mormon Spring to Preston Spring in partially insulated bottles. The temperature fell seven or eight degrees in the course of transfer, though this circum-

TABLE II

	Lot of fishes	Weight of sample	Number of fishes	Mean weight	ml./gm./hr.		
					Procedure 1	Procedure 2	Means
Mormon at Mormon	T	3.5	9	0.39	0.739	0.612	0.675
	U	4.4	6	0.73	0.597	0.555	0.571
	V	5.1	6	0.85	0.528	0.544	0.536
	W	8.3	6	1.38	0.382	0.423	0.402
	Means			0.84	0.561	0.533	0.546
Mormon at Preston (2½ to 4 hours)	T′	Same values			0.422		
	U′				0.374		
	V′				0.328	0.342	0.335
	W′				0.255	0.263	0.259
	Means				0.345		
(1 day)	T″	Same values			0.268	0.308	0.288
	U″				0.256	0.288	0.272
	V″				0.266	0.308	0.287
	W″				0.278	0.304	0.291
	Means				0.267	0.302	0.284

stance would seem to have little bearing on the results. Three different series of these fishes (8 lots, comprising 57 fishes) were thus transferred. The most instructive of these series consisted of the same four lots as were employed in the principal Mormon-at-Mormon tests. The further history of these fishes is included in Table II, and is portrayed graphically, in part, in Fig. 4. After the test in Mormon Spring, these fishes were left over night in the latter, in the same tubes (screened at the ends). They were then taken in bottles to Preston Spring and tested,

first, after the lapse of 2½ to 4 hours following transfer; next after a day. As regards their performance after this briefer period, we have complete figures only for the second of our procedures in computing oxygen consumption. The mean of these four values is 0.345, which is plainly intermediate between 0.561 (before the transfer) and 0.267 (one day following the transfer).

The significant features to be observed in the behavior of these four lots of fishes at the close of a day following transfer are (1) the great fall in metabolic rate in the cooler water; (2) the inverse correlation be-

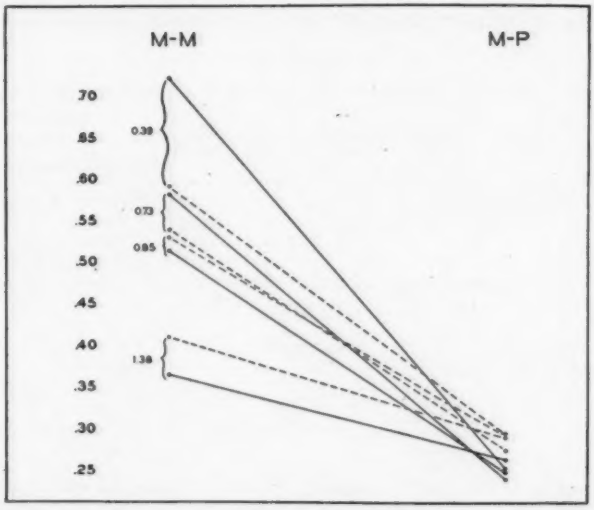

FIG. 4. Rate of oxygen consumption of same group of Mormon Spring fishes: (1) at Mormon Spring; (2) after 1 day at Preston Spring. Ordinates = ml./-gm./hr. Solid and broken lines have same significance as in two preceding figures. Figures opposite brackets denote mean weights of the fishes of these respective lots.

tween the size of the fishes and the rate of this fall, resulting in a marked convergence of the curves for the four size-groups, which come to nearly a common level by the end of 24 hours.

A question of considerable interest is whether the present figures give evidence that the respiratory metabolism of these warm-adapted fishes, when transferred to much cooler water, undergoes any such fall below the rate shown by cool-adapted specimens as had been shown in previous laboratory experiments.

At the close of the day our mean figure for the metabolic rate of six lots of Mormon-at-Preston fishes was 0.284; that for the Preston fishes in their natural habitat being 0.267. The mean weight of the Mormon-at-Preston fishes (0.79 gm.) is, however, much less than that of the Preston-at-Preston fishes (1.56 gms.), a fact which might, perhaps, account for this higher rate. Graphs for these two groups (Fig. 3), in which each is plotted according to weight, make it plain that any possible difference of metabolic rate, in either direction, is small in extent.

It cannot, therefore, be affirmed with any probability that these warm-spring fishes, when transferred to water at 21°, display a rate of oxygen consumption which is lower, at the close of one day, than fishes native to the latter. This conclusion may be regarded as confirming the results of the earlier test with the cyanide method, already referred to.

Two of the eight Mormon-at-Preston lots comprised in our various experiments were kept in the latter spring for two more days.[11] The figures for these two lots (mean weight 0.52 and 0.57 grams) were 0.283 and 0.343. While not very consistent, these figures certainly do not indicate any further fall of metabolic rate.

The convergence of the four pairs of curves in figure 4 is a striking feature of this graph and deserves some comment. At the close of a day in the cool spring, the differences between these four lots, in respect to metabolic rate, have virtually disappeared, or have even been slightly reversed. Thus, the correlation between size and metabolic rate, which is so conspicuous at 36°, has vanished at 21°. It has already been pointed out that this correlation is low in the cool-spring fishes themselves.

A marked divergence of the temperature-O_2-consumption curves for "large" and "small" *Fundulus* is shown by Wells (1935*a*, Figs. 3 and 4). Wells's observations relate to temperatures ranging from 10° to 24°, so that the case is not altogether parallel with our findings on *Crenichthys*, in which we are dealing with water at 21° and 36°, and with no intermediate values. In the case of our warm-spring fishes, too, the correlation actually disappears at the lower temperature. It is plain, however, that the two cases are in essential agreement in one important respect.

That the metabolic rate of organisms declines with age, or conversely, that a higher metabolic rate is to be expected in younger animals, appears to be an accepted fact (Child, 1915; Heilbrunn, 1937). Since fishes are known to grow more or less continuously through life, it is usually a safe assumption to regard relative size as an index of relative age. Our

[11] Some specimens were kept for four and even six days, but these were weighed in the meantime. Since a number of these fishes died as a probable result of the unavoidable drying, the results cannot be regarded as significant.

series of fishes of increasing weight (Table I) may therefore be safely regarded as representing, on the whole, a series of increasing age.

Concerning the correlation between age and the temperature-coefficient, less appears to be known. The majority of workers seem to have reported that this coefficient increased with age (Bělehrádek, 1935). Our figures, on the contrary, as well as those of Wells, certainly indicate a higher temperature coefficient for the *smaller* fishes.

Summary

Fishes (*Crenichthys baileyi*) living in a warm spring at temperatures of 35° to 37° have a far higher rate of oxygen consumption (ml./-gm./hr.) than fishes of the same species living in a cool spring (21°). The ratio, when fishes of approximately the same weight are compared, is not far from 2 to 1.

Cool spring fishes, when transferred to the warm spring, die so rapidly that measurements of oxygen consumption are not practicable.

When the converse experiment is performed (transfer of warm-spring fishes to the cool spring), they live in health for some days, and may perhaps do so indefinitely. Changes in their rate of oxygen consumption are as follows: (1) A rapid fall occurs within the first few hours. (2) At the close of a day, the metabolic rate (oxygen consumption) is about the same as that of fishes native to the cool spring. If any difference exists, when specimens of the same size are compared, this is probably slight.

This failure of the metabolic rate of warm-spring fishes to fall below that of cool-spring fishes, when transferred to the latter medium, accords with previous experiments at these same springs in which the cyanide method was used, but it does not accord with experiments performed upon some other species in the laboratory.

A marked inverse correlation exists between size (age) and metabolic rate. This correlation is more marked at high temperatures than at lower ones. Thus the curves for the four size-groups of warm-spring fishes here considered converge strongly during the test in cool water, coming together at nearly a common point by the end of the day. This last fact harmonizes with the fact that the correlation between size and metabolic rate, while observable, was found to be low among the cool spring fishes.

The oxygen consumption of smaller fishes is much more affected by temperature changes than is that of the larger specimens. In other words, the temperature coefficient is higher for the smaller (younger) animals, a fact which does not appear to agree with most results which have been reported for other organisms.

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